Structure-function characterization of SRMS: Validation of Dok1 as a SRMS substrate

A Thesis Submitted to the College of

Graduate Studies and Research

In Partial Fulfillment of the Requirements For

The Degree of Master of Science

In the Department of Biochemistry

University of Saskatchewan,

Saskatoon

By

Raghuveera Kumar Goel

(SUPEVISOR: Dr. Kiven Erique Lukong)

PERMISSION TO USE

In presenting this thesis in partial fulfillment of the requirements for a Postgraduate degree from the University of Saskatchewan, I agree that the Libraries of this University may make it freely available for inspection. I further agree that permission for copying of this thesis in any manner, in whole or in part, for scholarly purposes may be granted by the professor or professors who supervised my thesis work or, in their absence, by the Head of the Department or the Dean of the College in which my thesis work was completed. It is understood that any copying or publication or use of this thesis or parts thereof for financial gain shall not be allowed without my written permission. It is also understood that due recognition shall be given to me and to the University of Saskatchewan in any scholarly use which may be made of any material in my thesis.

Requests for permission to copy or to make other use of material in this thesis in whole or part should be addressed to:

Head of the Department of Biochemistry

University of Saskatchewan

Saskatoon, Saskatchewan S7N 5E5

ABSTRACT

SRMS (Src-Related tyrosine kinases lacking C-terminal Regulatory tyrosine and N terminal Myristoylation Sites) belongs to a family of non-receptor tyrosine kinases, which also includes breast tumor kinase (BRK). *SRMS* was first identified in 1994 in a screen for the genes that regulate the growth and differentiation of neuroepithelial cells. This 54 kDa protein spanning 488 amino acids, consists of the prototypical Src homology 3 (SH3), Src homology 2 (SH2) and a tyrosine kinase domain. While BRK has been documented for its expression in over 60 % of breast carcinomas, information on SRMS on similar grounds remains absent from the literature. Furthermore, unlike BRK, knowledge of how SRMS regulates its enzymatic activity as well as the identification of its substrates remains unknown. The work in this thesis demonstrates that SRMS is potentially expressed in the majority of breast carcinomas. To understand the biochemical and cellular functions of SRMS, a series of mutants comprising point mutations as well as the deletion of the N-terminal region and the functional, SH3 and SH2 domains, were generated and assessed for enzymatic activity in cells. This study demonstrates for the first time that the wild type protein is apparently constitutively active and that its N-terminal region regulates its enzymatic activity. As well, three critical amino acid residues in the protein namely, lysine 258 (ATP binding site), tyrosine 380 (auto-phosphorylation site) and tryptophan 223 (intramolecular interaction) have been characterized. All three residues have been determined to be essential for the enzymatic activity of SRMS. Finally, the adapter protein Dok1 has been characterized as a novel substrate of SRMS. The results from the present study underscore the potential significance of the catalytically active non-receptor tyrosine kinase, SRMS that should serve as a foundation upon which further research may ensue in the context of breast tumorigenesis.

ACKNOWLEDGMENTS

I deem it invaluable to thank my supervisor and mentor, Dr. Kiven Erique Lukong for his guidance, and perseverance towards the completion of my MSc program. I extend my gratitude to the present and former members of our lab, Chenlu Dai, Sayem Miah and Lexie Martin. I greatly appreciate the assistance of the Department secretaries, Mrs. Margaret Strohan and Mrs. Lori Lisitza throughout the entire tenure of my graduate program. I also thank the members of my supervisory committee, Dr. William J. Roesler (Department Head), Dr. Stanley Moore (Department Graduate Chair) and Dr. Deborah Anderson (Director of research, Saskatchewan Cancer Agency). And it is but in no less measure that I also extend my gratitude to Mr. James Talbot, Department of Biochemistry.

I cherish and laud the priceless support and faith bestowed upon me by my family and Dr. Lukong, alike.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ADAM/Adamalysin	A Disintegrin and Metalloproteinase
AEBSF	4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride
APS	Ammonium Persulfate
AP2	Activating Protein2
ARAP1	Ankyrin Repeat and Pleckstrin homology containing domain 1
ASK1	Apoptosis signal-regulating kinase 1
BCR-ABL	Breakpoint Cluster Region- Abelson Murine leukemia viral oncogene homolog 1
BRK	Breast tumor Kinase
BKS	BRK Kinase Substrate
BSK	β-cell Src homology Kinase
c-Abl	c-Abelson Murine Leukemia viral oncogene homolog 1
cAMP	Cyclic Adenosine Monophosphate
CDC2	Cell Division Cycle protein 2
CDK4/5/6/7	Cyclin Dependant Kinase 4/5/6/7
A/B/c-Raf	A/B/c-Rapidly Accelerated Fibrosarcoma
CREB	cAMP Response Element-Binding protein
CRK	CT-10 Related Kinase
CRK II	CT-10 Related Kinase II
DAB	3,3′-diaminobenzidine tetrahydrochloride
DAPI	4,6′-diamidino-2-phenylindole

Dok1	Downstream of Tyrosine kinases 1
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethylsulfoxide
DTT	Dithiothreitol
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
ERK1/2/	Extracellular signal Regulated Kinase 1/2/5
FAK	Focal Adhesion Kinase
FITC	Fluorescein isothiocyanate
FOXO1	Forkhead box protein O1
FRK	Fyn-Related Kinase
GABA	Gamma-Aminobutyric Acid
GPCR	G Protein Coupled Receptor
GTPase	Guanosine Triphosphatases
GST	Glutathione S-Transferase
GFP	Green Fluorescent protein
GSK3β	Glycogen Synthase Kinase 3 Beta
GTK	Gut Tyrosine Kinase
HEK293	Human Embryonic Kidney 293
HGF	Heregulin growth factor
HRP	Horse Radish Peroxidase
IGF	Insulin-like Growth Factor

IGF-1R	Insulin-like Growth Factor 1 Receptor
I-FABP	Intestinal-Fatty Acid Binding Protein
IYK	Intestinal tyrosine Kinase
JAK	Janus Kinase
JNK 1/2/3	c-Jun N-terminal Kinases
KIR	Kinase Inhibitory Region
MAPK	Mitogen Activated Protein Kinase
MAPKK	Mitogen Activated Protein Kinase Kinase
MAPKKK	Mitogen Activated Protein Kinase Kinase Kinase
MDM2	Mouse Double Minute 2 homolog
MEF2	Myocyte Enhancer Factor-2
MEKK 1/2/3/4	MEK Kinase 1/2/3/4
MKK 3/4/6	Mitogen-activated Protein Kinase Kinase 3
MLK3	Mixed Lineage Kinase-3
MOPS	3-(N-morpholino)propanesulfonic acid
MSK 1/2	Mitogen and Stress activated protein Kinase 1/2
mTOR	Mammalian Target of Rapamycin
NFkB	Nuclear Factor Kappa Beta
P130 CAS	P130 CRK Associated Substrate
PBS	Phosphate Buffered Saline
PEI	Polyethyleneimine
PTEN	Phosphatase and Tensin Homolog deleted on chromosome 10
PTK	Protein Tyrosine Kinase

PTK5	Protein Tyrosine Kinase 5
PTK6	Protein Tyrosine Kinase 6
PTK70	Protein Tyrosine Kinase 70
PMSF	Phenylmethanesulfonyl fluoride
PΔN SRMS	Partial N-terminal Deletion SRMS
RSK	Ribosomal s6 Kinase
Sam68	Src-Associated in Mitosis, 68 kDa
SH2	Src-homology 2
SH3	Src-Homology 3
SHB	Src Homology 2 domain containing adapter protein B
SLM1/2	Sam68-Like Mammalian protein 1/2
SOCS3	Suppressor of Cytokine Signalling 3
SRMS	Src Related tyrosine kinase lacking C-terminal Regulatory tyrosine and N-terminal myristoylation sites
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
ΔSH3-SRMS	SH3 deletion SRMS
ΔSH2-SRMS	SH2 deletion SRMS
SMAD4	Mothers Against Decapentaplegic homolog 4
Sp1	Specificity Protein1
STAP-2	Signal Transducing Adapter Protein-2
STAT1/3/5	Signal Transducer and Activator of Transcription 1/3/5
TCF	T-Cell Factor
TGFβ	Transforming Growth Factor Beta
TPL2 kinase	Tumor Progression Locus 2 kinase

TrkA/B/C	Tropomycin Related Kinase A/B/C

1. Review of the Literature

1.1 Tyrosine kinases:

Protein Tyrosine kinases (PTKs) comprise a distinct cohort of enzymes that function to phosphorylate the tyrosine residues on other proteins or alternatively, those that lie within their own sequences, by auto-phosphorylation. Such phosphorylation is orchestrated by the catalytic/kinase domain itself and is accomplished by the covalent attachment of a phosphoryl group, derived from ATP, onto the tyrosine residues of the protein (Cox *et al.*, 2008). PTKs are known to regulate a plethora of cellular functions including cell growth and proliferation. Tyrosine phosphorylation serves as a vital stimulus to initiate a cascade of intra-cellular signalling pathways that culminate in events such as the regulation of gene expression in the nucleus (Radha *et al.*, 1996). Tyrosine kinases may broadly be categorized as receptor and non-receptor kinases. The human genome is known to encompass at least 90 tyrosine kinases which include 32 non-receptor tyrosine kinases, organized into 10 subfamilies, and 58 receptor tyrosine kinases distributed in 20 subfamilies (Robinson *et al.*, 2000). While receptor tyrosine kinases, like the epidermal growth factor receptor, are transmembrane protein receptors and usually serve as binding sites for extracellular ligands, non-receptor tyrosine kinases are intracellular and regulate activities downstream of their surface-based counterparts.

1.1.1 Cell signalling pathways:

Cells possess the biological ability to communicate with each other through a class of proteins known as receptors. A broad physiological class of such receptors exists that help transmit signals, and hence enable communication, between cells. Such receptors may be membrane-based (EGFR, TGFβ, Integrins etc.) or intracellular (steroid hormone receptors etc.) and are activated by ligands which are usually small molecules or peptide hormones such as EGF, FGF, VEGF and cytokines. Receptor activation as a result of ligand binding may either directly elicit a cell's physiological response (as in the case of the GABA receptor in neurons) or may initiate a cascade of intracellular signalling events before such a physiological response is produced. In most cases, a cell's functional output is the result of an interplay of such intracellular signalling events. A variety of intracellular signalling cascades, organized in a complex network comprising several proteins, exist within various cells. Furthermore, these signalling cascades may also cross-communicate with several proteins and thus tend to cross-activate signalling molecules from other cascades.

Some of the major signalling pathways involving the FRK/PTK6 family kinases shall be described in the subsequent sections.

1.1.1.1 MAPK pathway

The mitogen activated protein kinases are serine/threonine kinases that are widely conserved amongst eukaryotes and are known to regulate cellular processes pertaining to proliferation and differentiation as well as cell survival and cell death (Pimienta and Pascual, 2007). As shown in Figure 1.1, the MAPK signalling pathway is hierarchically organized into a three-tiered signalling module that begins with the activation of MAP3 kinases (MAP3K) through the activating stimuli from the cell membrane receptors. Activated MAP3K phosphorylates, and hence activates, MAP2K which in turn activates MAP kinase. MAPK, upon activation, may enter the nucleus and regulate gene expression to produce the appropriate cellular response (Pimienta and Pascual, 2007).

In contrast to the relatively linear mode of phosphorylation-dependent activation of the MAPK and MAP2K, MAP3Ks have a more complex mechanism of activation. Several MAP3Ks such as c-Raf, MEKK4 and MLK3 natively exist in a conformationally inactive or autoinhibited state that requires multiple steps for activation (Miyake *et al.,* 2007; Rajakulendran *et al.,* 2009). The activation of MAP3Ks essentially begins with the translocation of these proteins to the membrane where activated small G-proteins relieve the autoinhibitory constraints on the MAP3Ks. The conformationally open MAP3Ks then undergo homo or hetero-dimerization that subsequently permits the dimers to phosphorylate each other on their kinase domains, thereby rendering the MAP3Ks active.

The signalling pathways of the MAPK system that adopt the three-tiered hierarchy are described below.

1.1.1.1.1 ERK1/2 pathway:

Perhaps the most well-characterized of the three pathways, the ERK1/2 pathway receives activating stimuli from a variety of cell surface receptors including receptor tyrosine kinases, integrins and ion channels (Zhang and Dong, 2007) (Fig 1.1). The key MAP3Ks of this pathway include A-Raf, B-Raf and c-Raf as well as Mos and TPL2 kinases. Activation of these proteins allows for the phosphorylation and activation of MEK1/2 which further activates ERK1/2. ERK1/2 can then translocate to the nucleus where it phosphorylates a number of substrates that regulate gene expression. This eventually regulates processes such

as cell proliferation, cell cycle progression, cell division and differentiation. Some of the ERK substrates include the RSK-related kinases, MSK1 and MSK2 as well as transcription factors like AP-1 (Zhang and Dong, 2007).

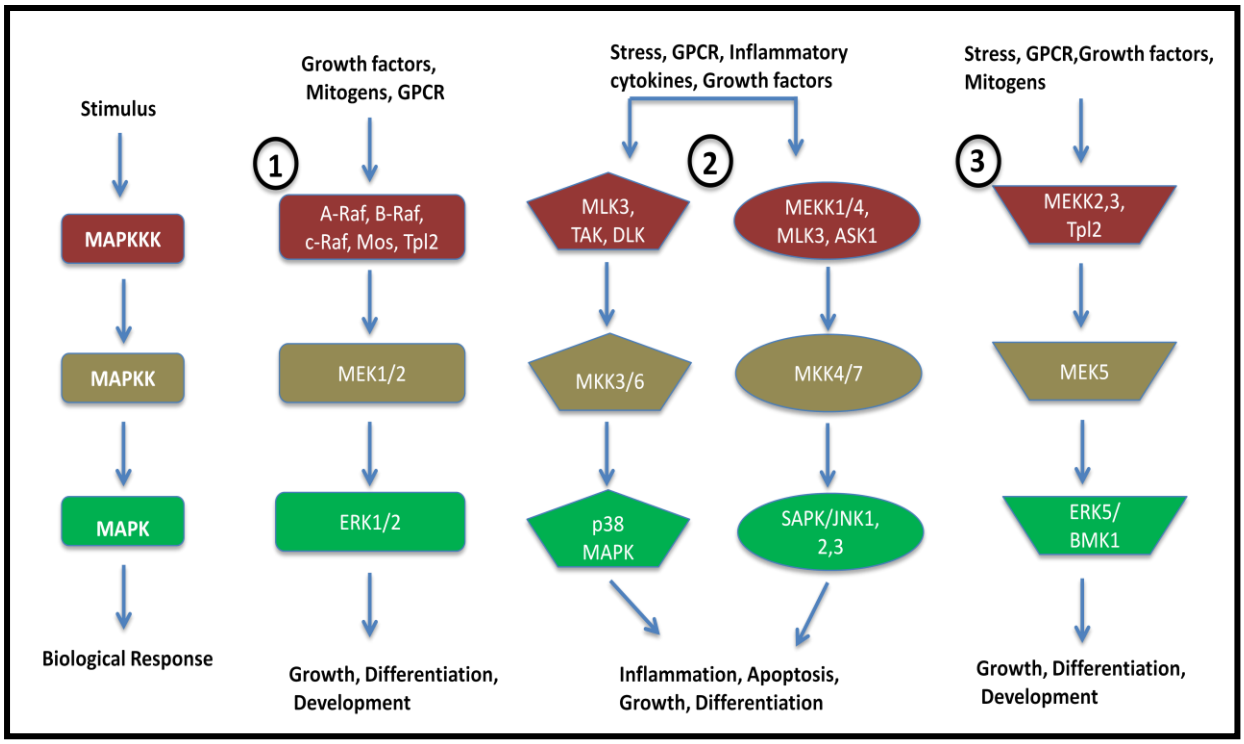

FIGURE 1.1: **Schematic diagram of the three-tiered MAPK pathways**. MAPKKK activation, following ligand stimulation, leads to the activation of MAPKK which further activates MAPK to allow for its nuclear translocation and the ultimate activation of transcription factors to initiate gene transcription and elicit a biological response. The three cellular pathways conforming to this hierarchical organization are shown: 1. ERK1/2 pathway, 2. p38/JNK pathway and 3. ERK5 pathway

1.1.1.1.2 JNK/p38 pathways:

Unlike the ERK1/2 pathway, the JNK/p38 pathways are activated in response to environmental stresses as well as inflammatory cytokines, growth factors and GPCR. Specifically, the MAP3Ks of the p38 pathway include MLK3, TAK and DLK while those of the JNK include MEKK1/4, MLK3 and ASK1 (Weston and Davis, 2002; Zarubin and Han, 2005) (Fig 1.1). Although apparently discrete, the JNK and the p38 pathways are often found to be functionally associated. Such co-regulation may be attributed to functioning of certain MAP2Ks, such as MKK4, that are common downstream activators of both pathways. The JNK and p38 pathways are, thus, often found to be co-activated (Cargnello and Roux, 2011). Other corresponding MAP2Ks that activate p38 and JNK1/2/3 are MKK3/6 and MKK7, respectively. Activated p38 and JNK kinases then translocate to the nucleus to phosphorylate an array of transcription factors such as Pax6, p53, STAT1, Max, Myc, ELK1, SMAD4, c-Jun and STAT3, leading to gene transcription and generating cellular responses pertaining to inflammation, apoptosis, growth and differentiation (Weston and Davis, 2002; Zarubin and Han, 2005).

1.1.1.1.3 ERK5 pathway:

The ERK5 pathway is also activated by a variety of stimuli such as cellular stress, growth factors, mitogens and GPCR. ERK5, also referred to as BMK1, is solely activated by MEK5 (Zhang and Dong, 2007) (Fig1.1). MEK5 as well as its upstream activator MEKK2/3 possess an N-terminal PB1 domain that permits direct heterodimerization between the two enzymes which thus also defines the specificity of such interactions. The PB1 domain on MEK5 also enables interactions with and hence activation of ERK5. Activated ERK5 translocates to the nucleus whereby several transcription factors like c-Fos, MEF2, CREB etc. are activated to induce gene transcription (Turjanski *et al.*, 2007). Besides regulating cell growth and differentiation, ERK5 is also known to be involved in mammalian vasculogenesis and endothelial barrier functions (Hayashi and Lee, 2004; Regan *et al.*, 2002).

1.1.1.2 JAK/STAT pathway:

A major pathway of the white blood cells, the JAK/STAT pathway, is known to transmit chemical signals from a variety of growth factors and cytokines to initiate gene transcription. JAKs bind to certain transmembrane receptors such as the cytokine receptors. Ligand binding to the cytokine receptors leads to the activation of the bound JAKs which

phosphorylates tyrosine residues on the receptor (Hebenstreit *et al.*, 2005) (Fig1.2). These phosphorylated tyrosine residues then serve as binding sites for SH2 domain containing proteins such as STAT. There are seven identified STAT proteins in mammals. Upon binding to the receptor, STAT proteins are tyrosine phosphorylated by JAK which results in hetero/homo dimerization of the STAT proteins. Such active STAT dimers then translocate to the nucleus where they initiate gene transcription. Nonetheless, STAT activation via other receptor tyrosine kinases like EGFR or non–receptor tyrosine kinases like Src, also leads to similar outcomes (Hebenstreit *et al.*, 2005). Also, multiple avenues exist for the negative regulation of the JAK/STAT pathway. For instance, tyrosine phophatases such as SHP1 or certain STAT competitors like SOCS which compete with STAT for binding to the cytokine receptors mediate feedback inhibition of the JAK/STAT signalling pathway (Krebs and Hilton, 2001; Xu and Qu, 2008). The JAK/STAT pathway is known to affect cellular processes such as growth, proliferation, immune response and haematopoiesis (Hebenstreit *et al.*, 2005).

1.1.1.3 PI3K/AKT pathway:

The activation of the PI3K/AKT cascade begins with signals transmitted from the cell surface receptors like the receptor tyrosine kinases, T and B cell receptors, integrins and cytokine receptors that trigger the production of phosphatidylinositol 3,4,5 triphosphate (PIP_3) from phosphatidylinositol 4,5 bisphosphate (PIP_2) via PI3 kinase (PI3K) (Carnero *et al.*, 2008) (Fig 1.3). Such lipids serve as plasma membrane docking sites for the Pleckstrin Homology (PH) domain-containing proteins such as AKT (also known as Protein kinase B or PKB) and PDK1 (Carnero *et al.*, 2008). Binding to the plasma membrane positions AKT for phosphorylation by its activating kinases; mTORC2 and PDK1 which phosphorylate AKT at S473 and T308, respectively. Once activated, AKT triggers the activation of several other cellular proteins including, but not limited to, mTORC1 and MDM2 (Manning and Cantley, 2007). Active mTORC1 phosphorylates and activates the 4E binding protein 1 (4E-BP1) and p70S6 kinase to initiate protein translation leading to cell growth. AKT-mediated phosphorylation of MDM2 results in the degradation of the pro-apoptotic p53 protein which leads to cell survival (Manning and Cantley, 2007). FOXO1 is another AKT target which upon phosphorylation is sequestered to the cytoplasm and effectively inhibited from exerting its transcriptional activity. FOXO1 is known to regulate the transcription of several genes involved in apoptosis, DNA repair and cell cycle arrest. GSK3β is also inhibited by AKT

which leads to the translocation of β-catenin to the nucleus where it enhances gene transcription of genes like cyclin D1 and c-myc, known to initiate the progression of cell cycle from the G1 to S phase (Dal Col and Dolcetti, 2008; Zhang *et al.*, 2012). Thus, the PI3K/AKT pathway is intrinsically linked to the regulation of cell growth, proliferation as well as cell survival (Tzivion *et al.*, 2011). Signalling through AKT may be downregulated via PTEN that essentially dephosphorylates PIP_3 to yield PIP_2. Thus PTEN is also considered a tumor suppressor (Georgescu, 2010). Direct dephosphorylation of AKT via such phosphatases as PHLPP 1/2 also serves as a mechanism of negative regulation of the pathway (Brognard and Newton, 2008).

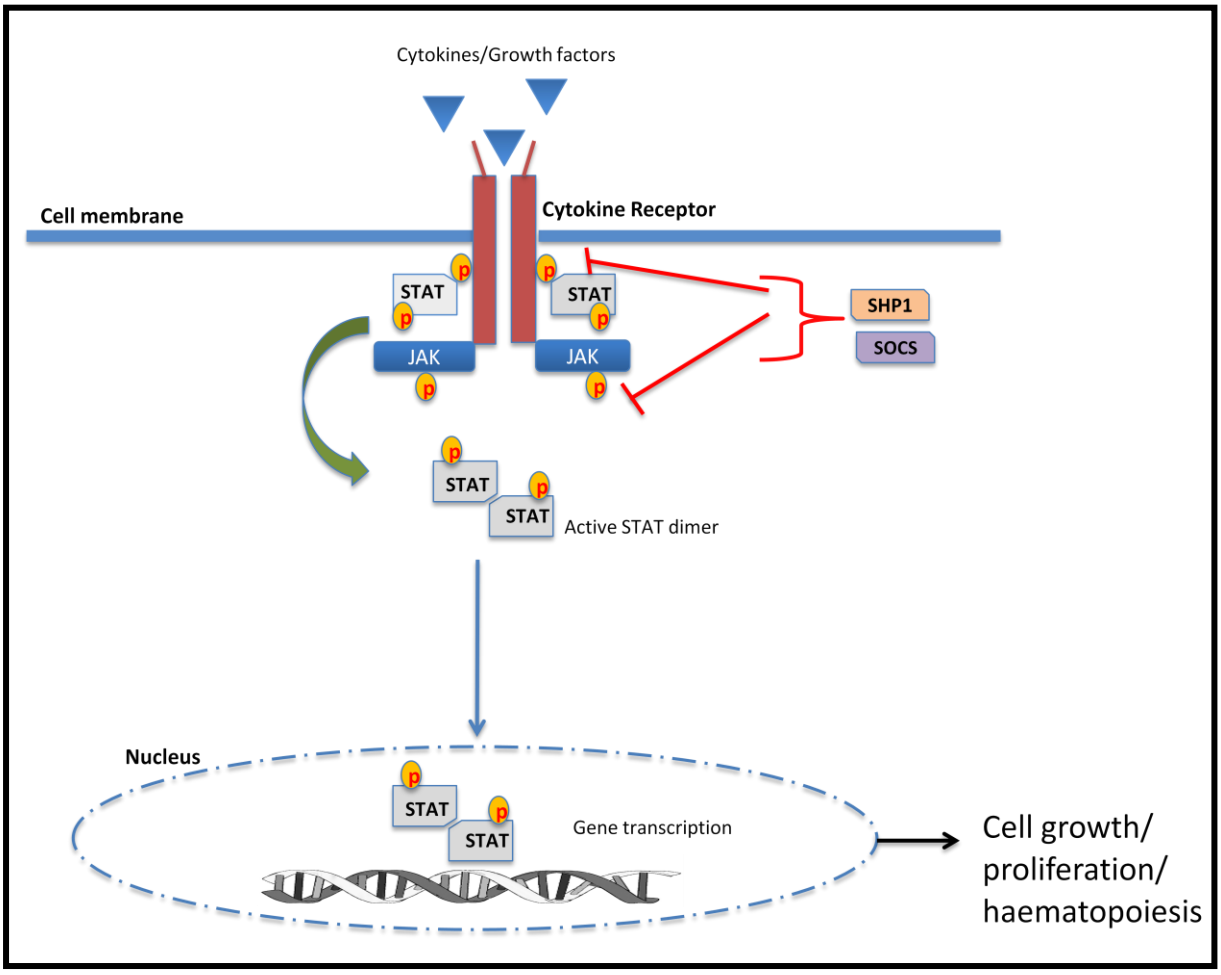

FIGURE 1.2: Schematic representation of the JAK/STAT pathway. Ligand stimulation, via growth factors or cytokines, triggers the autophosphorylation and hence the activation of the JAK kinases bound to cytokine receptors. Activated JAK kinases phosphorylate the cytokine receptors. Tyrosine phosphorylated sites on the receptor serves as binding sites for STAT proteins which, upon binding, are in turn phosphorylated by JAK kinases. Upon phosphorylation, STAT molecules undergo dimerization and nuclear translocation where the

activate dimers initiate gene transcription. Negative regulation of the JAK/STAT pathway is mediated by the actions of proteins like SOCS and SHP1.

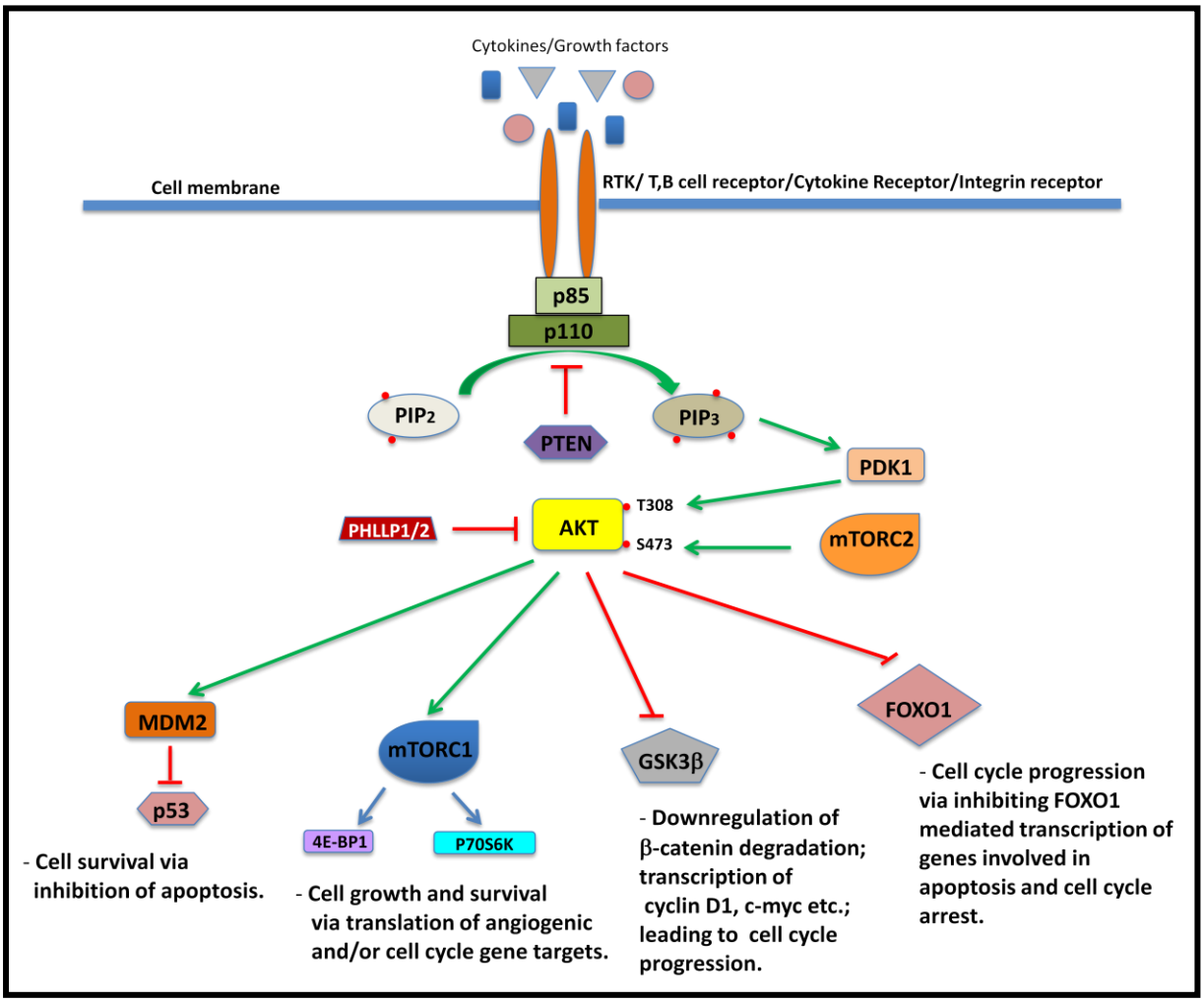

FIGURE 1.3: Schematic representation of the PI3K/AKT pathway. Ligand stimulation, via different growth factors or cytokines, triggers the production of PIP_3 from PIP_2 via PI3K activity. The docking of PDK1 to PIP_3 activates PDK1 which phosphorylates AKT on T308 which together with S473 phosphorylation via mTORC2 fully activates AKT. AKT activation then leads to the activation and/or inhibition of several downstream targets to promote cell survival, growth and proliferation. Shown here are some of the downstream effectors of AKT; MDM2, mTORC2, GSK3β and FOXO1.

1.1.2 Src- Family Kinases (SFKs):

The Src family kinases comprise a prominent class of non-receptor tyrosine kinases, whose family members include: Src, Yes, Fyn and Fgr forming the SrcA subfamily and Lck, Hck, Blk and Lyn in the SrcB subfamily (Hanks and Hunter, 1995; Neet and Hunter, 1996). While v-Src was one of the first viral onco-proteins studied with a well characterized tyrosine kinase activity (Smart *et al.*, 1981), its cellular orthologue, c-Src was later identified in mammalian cells and shown to be proto-oncogenic (Oppermann *et al.*, 1979). c-Src maps to two chromosomal loci in humans, chromosome 1p36 and 20q13, both of which remain prone to genetic re-arrangements in malignancies (Le Beau *et al.*, 1985; Parker *et al.*, 1985). The coding transcript of c-Src comprises eleven exons, a splicing pattern that remains highly conserved across the Src family hierarchy (Cetkovic *et al.*, 2004; Kaplan *et al.*, 1987) (Fig 1.4). All SFKs share three conserved functional domains: a poly-proline-binding Src homology 3 (SH3) domain, a phosphorylated tyrosine-binding Src Homology 2 (SH2) domain and a tyrosine kinase domain (Fig 1.1). All Src family kinases also contain either a myristoylation or palmitoylation signal in the N-terminus that mediates anchorage to the plasma membrane. Phosphorylation of C-terminal Y527 in chicken c-Src by c-Src kinase (CSK) allows for an intra-molecular interaction of the C-terminal tail of Src with its SH2 domain, thereby locking the enzyme in an inactive conformation. This auto-inhibited conformation is further stabilized by intra-molecular interactions between the SH3 domain and the poly-proline sequence situated in the linker region, flanked by the SH2 & the kinase domains (Cooper *et al.*, 1986; Liu *et al.*, 1993; Superti-Furga *et al.*, 1993; Xu *et al.*, 1997) (Fig. 1.4). Such an autoinhibitory state of Src was evidenced through other studies employing mutational analysis where deletions of either the SH3 or the SH2 domains resulted in mutants that exhibited substantially higher kinase activity as well as transforming potential as compared to the wild-type form (Seidel-Dugan *et al.*, 1992). Interestingly, such deletion mutants were demonstrated to be hypophosphorylated at Y527, a condition that was believed to account for the higher kinase activity of these mutants. Nonetheless, a later study demonstrated that despite induced phosphorylation at Y527 by c-Src kinase, these mutants did not exhibit reduced kinase activity. This therefore suggests that the presence of intramolecular interactions is essential for the maintenance of the autoinhibited state of the kinase (Okada *et al.*, 1993).

The residues of the SH2-kinase linker segment form a left-handed polyproline type II helix that is conformationally sandwiched between the SH3 domain and the N-terminal lobe of the kinase domain and has been shown to contribute to the negative regulation of kinase activity in Src (Xu *et al.*, 1997). Unlike Blk, Lyn and Lck, other members of the family, including c-Src, possess only a single proline residue within the SH2-kinase linker segment that deviates from the consensus PXXP type motifs required for binding to the SH3 domain (Fig 4.10). In c-Src the position of the other proline, in the PXXP arrangement, is occupied by Glutamine 253 that packs against tyrosine 90 and tyrosine 136 within the SH3 domain, thereby yielding a characteristic class II binding orientation (Xu *et al.*, 1997). Additionally, the existence of a highly conserved tryptophan in the kinase linker region is known to stabilize an inactive conformation as studied in the Src family member, Hck (LaFevre-Bernt *et al.*, 1998). Interestingly this tryptophan (W260) is positioned outside of the polyproline helix and interacts with the alphaC-helix of kinase domain, upon binding of the linker prolines to the SH3 domain, to sustain a catalytically inhibited state. Furthermore, mutating this tryptophan also displaces the SH3 and SH2 domains, allowing them a greater ligand-binding surface for mediating substrate interactions (LaFevre-Bernt *et al.*, 1998).

In contrast, phosphorylation on the Y419 residue, leaves the protein in an "open-conformation", otherwise referred to as the active conformation (Boerner *et al.*, 1996). Also, while substitution of the active site lysine residue; K298 (ATP contacting) with a methionine inactivates the catalytic activity of Src kinase (Luttrell *et al.*, 1996), the substitution Y527F yields a constitutively active variant of the protein (Kmiecik and Shalloway, 1987; Roskoski, 2004).

The underlying biochemical events triggering such autophosphorylation and the consequent activation of the enzyme are mediated via a defined conformational re-arrangement of the catalytic domain. The crystal structure of Src has allowed for the structural characterization of the mode of activation of the enzyme (Cowan-Jacob *et al.*, 2005; Roskoski, 2004; Xu *et al.*, 1999). The structures studied have identified Src family kinase domains as containing two structural lobes; (i) The smaller N-terminal lobe comprised of a five-stranded β-sheet and a crucial glutamic acid residue (at position 310 in human Src, and (ii) a larger C-terminal lobe that is as the primary site of interaction with substrate peptides and comprised primarily of α-helices.

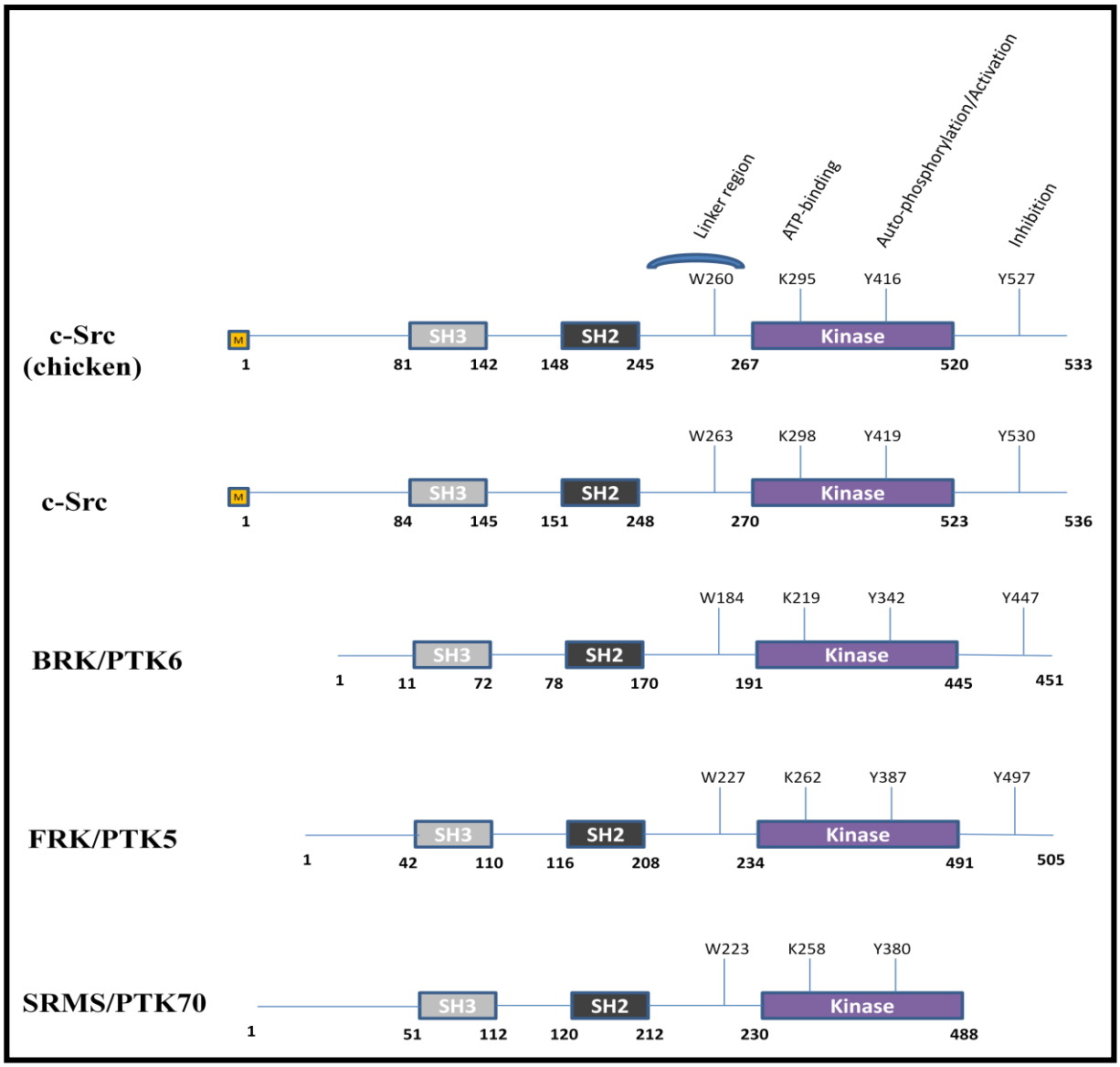

FIGURE 1.4: **Schematic representations of c-Src, and FRK/PTK6 family kinases (BRK, FRK and SRMS).** Src as well as FRK family kinases possess 3 functional domains, namely, the Src homology 3 (SH3), Src homology 2 (SH2) and the kinase domains. c-Src (both chicken and human orthologues) also possesses an N-terminal myristoylation signal. Shown here are the critical residues involved in the regulation of kinase activity of the enzymes. The autophosphorylation site in c-Src remains spatially conserved with that of BRK (Y342), FRK (Y387) and SRMS (Y380). Other residues that remain conserved include the carboxy terminus tyrosine residues, that negatively regulate catalytic activity in c-Src (Y530 and Y527 in chicken orthologue), BRK (Y447) and FRK (Y497), as well as the ATP-contacting lysine residues; K298, K219, K262 and K258, respectively. Also indicated are the highly conserved tryptophan residues within the SH2-kinase linker regions of these tyrosine kinases. These tryptophan residues have been implicated in the regulation active/inactive conformations of certain tyrosine kinases (LaFevre-Bernt *et al.*, 1998; Qiu and Miller, 2004).

Between these lobes lies the active site (Williams *et al.*, 1998). In the inactive state, the SH3 and SH2 domains remain associated with their respective intramolecular ligands (respectively, the polyproline sequence in the SH2-kinase linker segment and the regulatory tyrosine residue in the C-terminal tail region). These interactions also serve to regulate the conformation of the Y416 containing-activation loop located within the C-terminal lobe as well as the alphaC-helix loop of the N-terminal lobe. Typically, the SH3 and SH2 domains are conformationally stacked against the N and C-terminal lobes of the kinase domain, respectively (Fig 1.5). The resulting interactions of the SH3 and SH2 domains with their intramolecular ligands thus lock the N and C-terminal lobes in an inflexile conformation that allows the activation loop to form an inhibitory helix (Fig 1.5 and 1.6). The partially helical structure of the activation loop not only blocks the catalytic cleft of the kinase domain but also buries Y416, occluding access to ATP and thus preventing autophosphorylation. Thus, an inactive state of the PTK is maintained. However, the dephosphorylation of the C-terminal tyrosine, Y527, and/or the binding of the SH3 and SH2 domains to target peptides relieve the conformational constraints imposed on the PTK resulting in increased accessibility of the catalytic cleft to the ATP substrate, which essentially occurs with the disruption of the helical structure of the activation loop (Cowan-Jacob *et al.*, 2005; Roskoski, 2004; Xu *et al.*, 1999).

From a biochemical perspective, in the inactive state, the glutamic acid residue at position 310 interacts with an arginine at position 385, thereby stabilizing the alphaC-helix in an outward position which prevents the formation of a salt bridge with K295. The dephosphorylation of Y527 and/or the binding of the SH3 and SH2 domains to the target peptides results in autophosphorylation at Y416. Upon autophosphorylation, Y416 in the activation loop forms a salt bridge with R385, an event requiring the restructuring of the activation loop to a non-blocking conformation. This restructuring of the activation loop confers structural flexibility to the alphaC-helix so as to allow the latter to swing inwards, promoting the formation of the critical salt bridge between E310 and K295, thus activating the kinase activity of Src (Fig 1.6) (Breitenlechner *et al.*, 2005; Cowan-Jacob *et al.*, 2005; Xu *et al.*, 1999).

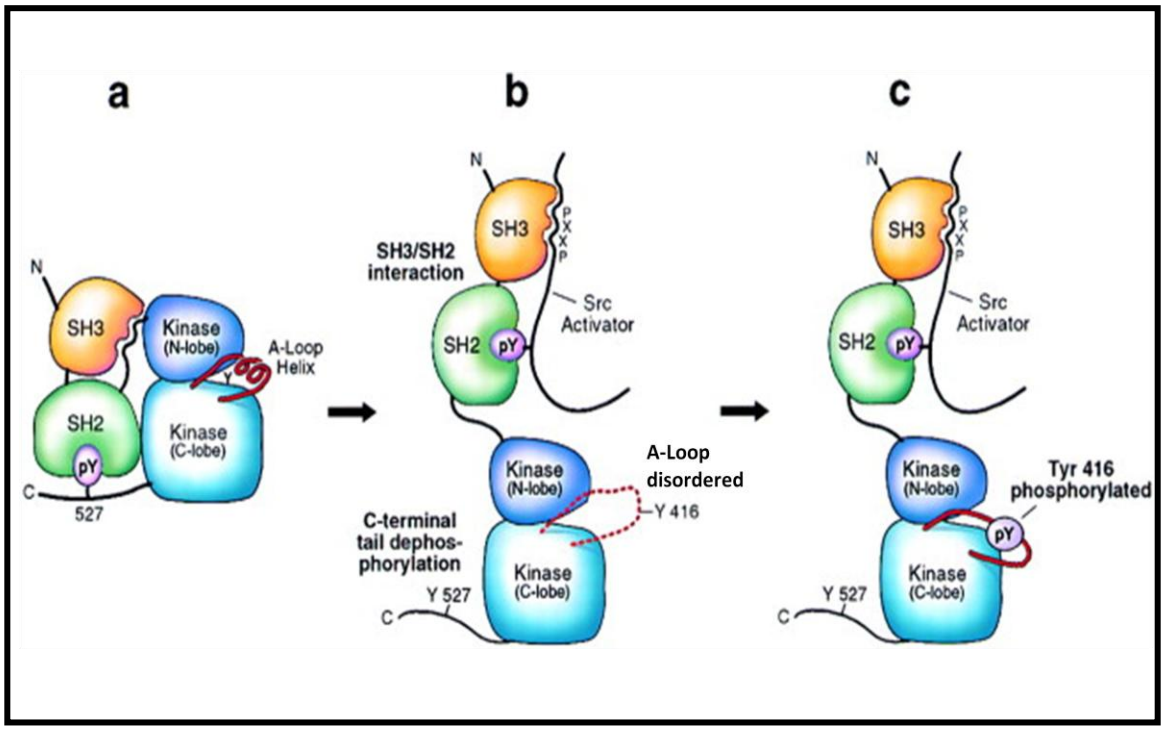

FIGURE 1.5: **Schematic representation of c-Src depicting the conformational shift from an inactive to an active state.** (a) The SH3 and the SH2 domains are packed against the kinase domain with the phosphorylated C-terminal tyrosine and the SH2-kinase linker region serving as intramolecular ligands for the SH2 and the SH3 domains, respecively. Consequently, a closed or inactive conformation is maintained with the inhibitory Activation-Loop (A-Loop) occluding access to ATP. (b) Upon the dephosphorylation of the C-terminal tyrosine and/or the binding of the SH3/SH2 ligands from target peptides, the intramolecular associations are relieved and the "closed" conformation changes to an "open" conformation. The A-Loop is disordered/destabilized and the autophosphorylation site is exposed. (c) Access to ATP results in the autophosphorylation of the tyrosine residue, thus activating the enzyme. (Figure obtained with permission from (Xu *et al.*, 1999)).

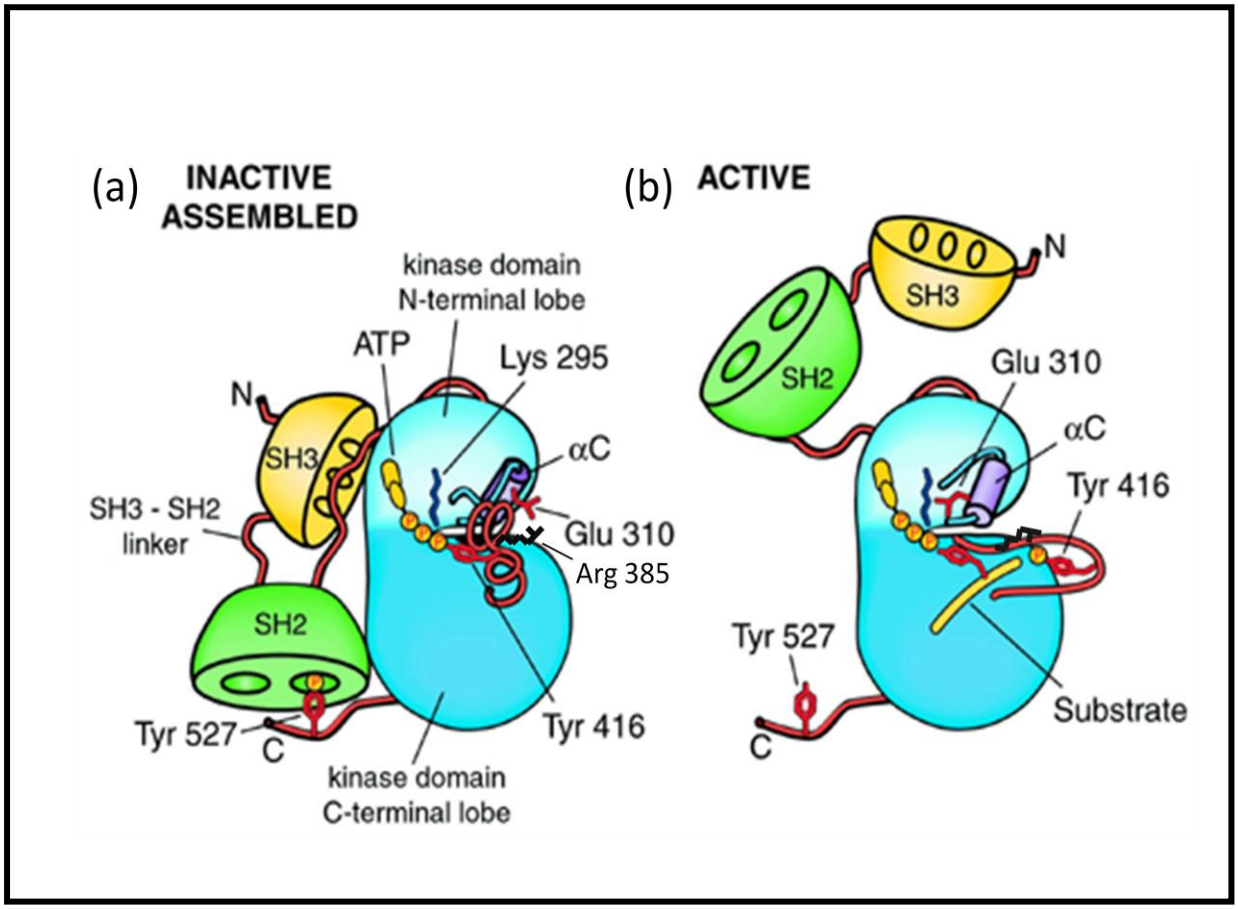

FIGURE 1.6: **Schematic representation of the biochemical activation of c-Src.** (a) In the inactive state, the binding of the SH3 and SH2 domains to their intramolecular ligands results in the conformational rigidity of both the kinase lobes. E310 within the N-terminal lobe forms a salt bridge with R385 which stabilizes an inactive position of the alphaC-helix. (b) The phosphorylation of Y416 triggers a conformational switch, promoting Y416 to form a salt bridge with R385 and E310 to salt bridge with K295, thus re-orienting the alphaC-helix in an inward position and activating Src. (Figure adapted form (Young et al., 2001).

1.1.1.1 Src SH3 domain

The Src family SH3 domains are involved in intramolecular as well as intermolecular interactions. These SH3 domains are typically conserved in most proteins and span approximately 50-60 amino acids. Such prototypical poly-proline-binding domains are known to be involved in the propagation of intracellular signalling cascades via interactions with proteins from several signalling networks (Pawson, 1994; Pawson and Gish, 1992). The SH3 domain quintessentially binds to short proline-rich motifs on target proteins/ligands in a type II helical conformation. Such motifs are typically sequenced X-P-X-X-P (where "P" refers to the Proline separated by two scaffolding residues "X") (Feng *et al.*, 1994; Lim *et al.*, 1994; Yu *et al.*, 1994). Furthermore, while two characteristic hydrophobic pockets on the SH3 domain's ligand-binding groove connects to the X-P dipeptides, a third "specificity pocket" contacts the residues flanking the conserved prolines, consequently governing the orientation of the resulting interaction. The "Specificity pocket" is as such negatively charged and interacts with a positively charged Arginine or Lysine on the ligand, facilitating two possible binding orientations; class I interactions wherein this typical residue lies N-terminal to the conserved prolines in the sequence +x-X-P-X-X-P or class II interaction with the residue being positioned C-terminal to the same, X-P-X-X-P-x+ (Feng *et al.*, 1995).

The SH3 domains fundamentally comprise two triple-stranded anti-parallel β sheets positioned at right angles to each other with certain conserved hydrophobic residues clustered together that participate in ligand-interaction (Erpel *et al.*, 1995; Musacchio *et al.*, 1992). Mutational analyses of the Src SH3 domain have identified key residues that are involved in ligand binding and recognition. Mutation of certain key residues in the two hydrophobic pockets of the chicken Src SH3 domain resulted in significantly reduced SH3-ligand interaction. Such residues include Y92, W118, P133, Y136 and Y90 (Weng *et al.*, 1995). In Lck, Y90 is replaced with H70, which may be reflective of subtle variations in the binding affinities of SH3 domains (Erpel *et al.*, 1995). Likewise, residues that govern ligand selectivity were identified via similar mutational studies. The third "specificity pocket" possesses two uniformly conserved residues, D99 and W118. Mutating D99 was specifically shown to reduce the binding of the mutant SH3 domain to p68 by 50% and exhibiting decreased associations with other SH3 binding proteins (Weng *et al.*, 1995). Other residues potentially involved in ligand selectivity are R95 and S94. Mutation of these residues resulted in an elevated specificity of the Src SH3 mutant towards binding to p85. Such mutations, however, resulted in decreased intramolecular interactions (Erpel *et al.*, 1995).

1.1.1.2 Src SH2 domain

The SH2 domains represent another class of highly conserved protein subunits that are found on a variety of signalling proteins dispersed across several signalling cascades (Grucza *et al.*, 1999; Sawyer, 1998). These domains functionally recognize and bind to phosphorylated tyrosine residues on target proteins (Songyang *et al.*, 1993). Structural delineation has been accomplished for a broad spectrum of proteins including several members of the Src family kinases, which reveal a conserved architecture for the SH2 domain as comprising a five stranded anti-parallel β-sheet core, clasped or sandwiched between two α-helices and extended by a β-strand and a small three-stranded β sheet spanning approximately 100 amino acids. Peptides bind perpendicular to the central five stranded anti-parallel β-sheet core. Such functional characteristics as ligand binding and selectivity have been attributed to two conserved pockets situated within the SH2 domains. Studies have shown that the phosphotyrosine binding pocket comprises several positively charged residues, most critical of which is aconserved arginine residue (R175 in v-Src) that forms a part of the SH2 "signature motif" FLVRES. Mutation of this arginine residue alone results in significantly reduced phosphotyrosine binding in Src (Campbell and Jackson, 2003; Tian and Martin, 1996). Another substitution, W148E, was also shown to impair phosphopeptide binding of the Src SH2 domain (Bibbins *et al.*, 1993). The second pocket required for ligand recognition/selection contains residues that are less conserved and vary across different SH2-containing proteins, thus conferring upon these proteins, distinct ligand-binding specificities (Campbell and Jackson, 2003). Earlier studies screening the Src family SH2 domains against phosphopeptide libraries with varying residues at the X1, X2 and X3 positions within the pY-X1-X2-X3 motif, revealed a strong selectivity for the pYEEI motif (Songyang *et al.*, 1993). Mutating a conserved threonine, identified as T215 in Src, to tryptophan has been shown to alter the specificity of the Src SH2 domain, resulting in an increased preference for a pYVNV ligand instead of the putative pYEEI ligand. Since the pYVNV motif serves as the preferred binding ligand for the Grb SH2 domain, the substitution, T215W, in the Src SH2 domain was, therefore, shown to biologically behave as the Grb SH2 domain (Pawson *et al.*, 2001).

More recently, a proteomics screen for phosphopeptides that bind to various Src family SH2 domains identified a characteristic pattern in the selectivity of the peptide motifs (Zhao *et al.*, 2013). Through the use of binding assays using a generic phosphopeptide motif, TAXXpYXXXLNBBRM (where "LNBBRM" represents the C-terminal linker conjugating the phosphotyrosine peptide to the beads and used towards facilitating peptide release and

mass spectral analyses), randomized for amino acid residues in the "X" positions, the Src SH2 domain was specifically shown to exhibit substantial preference for binding to peptides containing either a threonine or an alanine at the +1 position (Table 1.1). At the +2 position and +3 positions, specificity was significantly favoured towards a methionine and an isoleucine, respectively. However, at the -1 and the -2 positions, a tyrosine and a histidine residue was favoured, respectively (Zhao *et al.*, 2013). Interestingly, a significant divergence could be inferred between the SH2 domain specificities of Src and FRK/PTK6 family kinases. Specifically, while Src preferred threonine and methionine residues at the P+1 and P+2 positions of the phosphopeptides, respectively, FRK/PTK6 family kinases exhibited a relatively similar specificity towards glutamic acid and isoleucine residues at the same positions (Table 1.1).

1.1.2 FRK/PTK6 family Kinases

The FRK/PTK6 family kinases is comprised of four non-receptor tyrosine kinases namely, BRK (PTK6), FRK (PTK5), SRMS (PTK70) and Src42a/Dsrc41 with the latter being identified as a tyrosine kinase belonging to *Drosophila melanogaster* (Serfas and Tyner, 2003). These four tyrosine kinases have been clustered together in a single family owing to a significant similarity in their intron-exon structures. Like their Src family counterparts, members of the FRK/PTK6 family kinases are composed of the classic SH3, SH2 and the kinase domains (Serfas and Tyner, 2003). While the former two domains aid in the interaction of these kinases with other proteins targets, the latter confers a catalytic function to these, thereby defining their roles as intracellular enzymes and at the same time distinguishing these from the other classes of enzymes. The focus of the subsequent sections will be on BRK, FRK and SRMS.

The FRK/PTK6 family kinases lack myristoylation and palmitoylation signals, a unique feature that is critical for membrane association of Src family kinases. At the genomic level, BRK, FRK and SRMS are evolutionarily conserved. The fact that their intron-exon structures remain highly conserved with one another, and to a lesser degree with that of the Src family members, is a primary reason for their incorporation into a distinct family of non-receptor tyrosine kinases. All three tyrosine kinases possess 8 exons and reflect conserved splicing boundaries that differ from those of Src family kinases (Serfas and Tyner, 2003) (Fig 1.7).

TABLE 1.1 SH2 domain specificities of Src and FRK/PTK6 family kinases. The SH2 domain specificities of Src, BRK, FRK and SRMS as determined by Zhao et al. using phosphopeptide motif TAXXpYXXXLNBBRM (Zhao *et al.*, 2013)., Randomized residues are denoted by "X" .

NRTK	P-2	P-1	pY-0	P+1	P+2	P+3
Src	H	Y M F H	Y	T A N	M Q V I	I M
BRK/PTK6	H E N D	M Y H F	Y	D E	I N V M	C V Y
FRK/PTK5	H N W	F Y	Y	E C H	N I C D	I C L M
SRMS/PTK70	H W T C	H F Y W	Y	H E C	I Y C M	C F M V

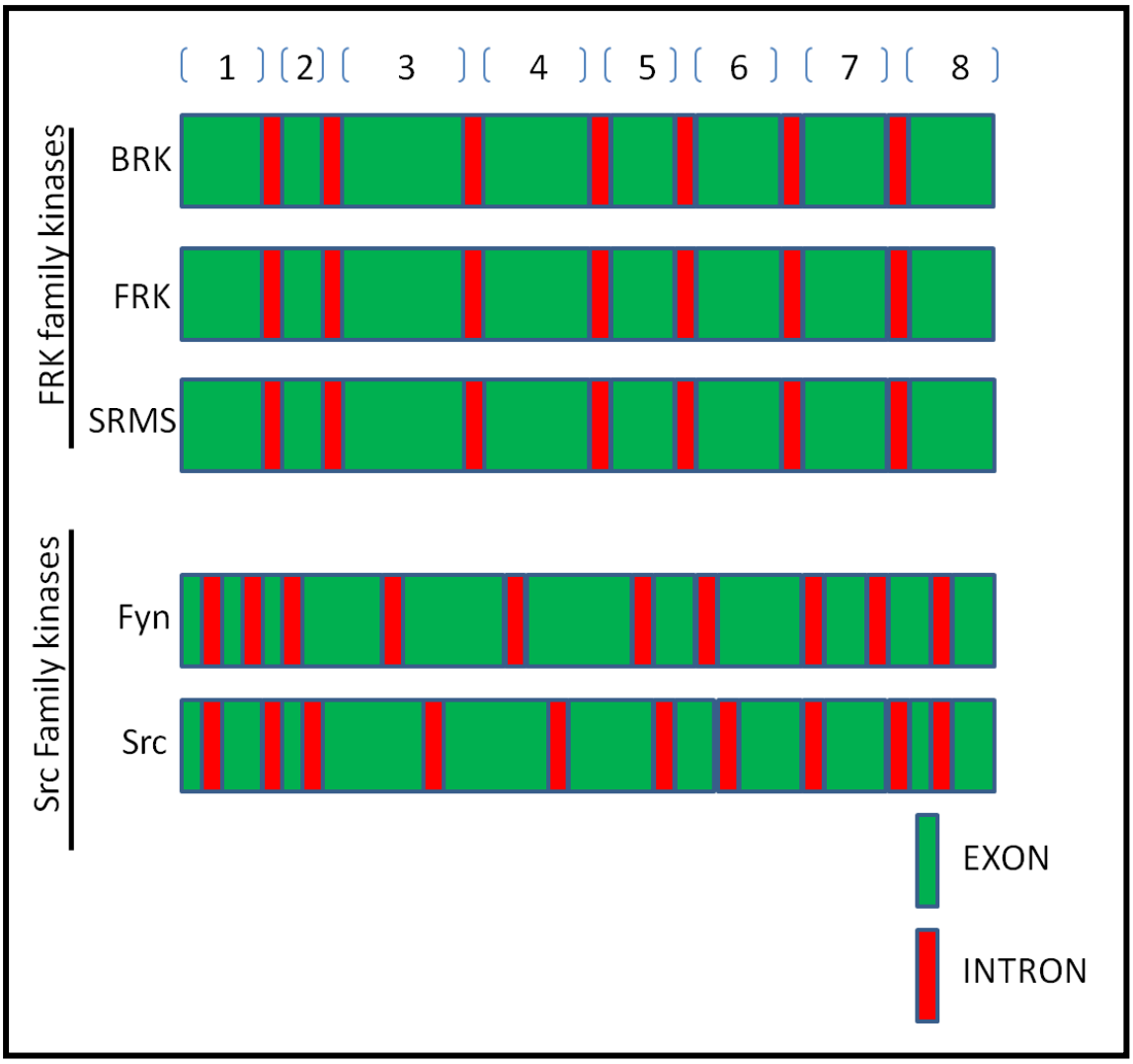

FIGURE 1.7: **Schematic representation of the Intron-Exon splicing pattern of the FRK family (BRK, FRK and SRMS) and Src family kinases**. The FRK family kinases possess 8 exons. This differs from the Src family kinases as shown for Fyn and Src kinases which have 11 exons. Thus, the FRK family not only displays a diverging splicing pattern but also an unequal number of introns and exons as compared to the Src family. (Figure adapted from (Serfas and Tyner, 2003)).

BRK and FRK have been extensively characterized for their structural and functional characteristics. Furthermore, several substrates/ binding partners have been identified and characterized for the two tyrosine kinases. However, the third member of the FRK/PTK6 family, SRMS, remains scarce to date. Indeed, information pertaining to its structural, biochemical and functional characteristics is either absent or limited. The same holds true for its substrate-specificity. Nonetheless, in recent published reports, efforts to determine the identity of the novel kinase's potential substrates (Takeda *et al.*, 2010) and, specifically, its SH2 domain recognition motifs (Zhao *et al.*, 2013), have yielded valuable information that can potentiate further biochemical and functional research on the kinase. The subsequent sections shall extensively review background information on BRK, FRK and SRMS.

1.1.2.1 Breast tumor kinase (BRK)

1.1.2.1.1 Gene discovery and organization

BRK, pronounced as "Berk", is also referred to by its other synonym, PTK6 whose murine ortholog is designated as Sik (Src-like intestinal kinase) (Lee *et al.*, 1993; Llor *et al.*, 1999). The gene was first identified as one amongst 25 other novel tyrosine kinases expressed in cultured human melanocytes that were surveyed via reverse transcriptase-polymerase chain reaction (Lee *et al.*, 1993). In subsequent studies, the full length BRK cDNA was identified and cloned from the breast carcinoma cell lines MCF7 and T47D (Mitchell *et al.*, 1994) and later, from a human intestinal cDNA library (Llor *et al.*, 1999). Through fluorescence *in situ* hybridization (FISH) analysis, the gene encoding BRK has been assigned to chromosome 20q13.3 in humans and chromosome 2 in mice (Llor *et al.*, 1999; Park *et al.*, 1997). The BRK transcript is composed of eight exons however only the boundaries between exons 1/ 2 (separating exons coding for the SH3 and the SH2 domains) and 7/ 8 (separating the exons encoding the C-terminal region of the kinase domains) remain conserved with members of the Src family (Lee *et al.*, 1998; Mitchell *et al.*, 1997) (Fig 1.4). This becomes particularly interesting since BRK otherwise shares nearly 44% amino acid sequence identity with members of the Src family. Furthermore, of the total seven BRK exon boundaries, six are conserved with the structurally related *Drosophila* Src gene, Dsrc41, which itself possesses nine exons. This presents clues about both the genes sharing a common evolutionary ancestor or origin (Mitchell *et al.*, 1997). All in all, the genetic locus of BRK spans about 10

kb in comparison to the 20 kb region held by Src (Anderson *et al.*, 1985; Mitchell *et al.*, 1997).

The human BRK gene also encodes an alternate transcript that is spliced differentially. Such a transcript, designated ALT-PTK6 and originally referred to as Lambda m5, encodes a 134 amino acid (15 kDa) protein comprising only the N-terminal region fused with the SH3 domain and a unique C-terminal tail abundant in proline residues. A consequence of alternative splicing, such an event leads to the omission of exon 2 thereby causing a shift in the open reading frame and a termination 172 bp 3′ of the transcript (Brauer *et al.*, 2011; Mitchell *et al.*, 1997).

Preliminary analysis of the BRK gene promoter revealed a region roughly 800 bp upstream of the translation start site to contain nearly 60% of the SV40 promoter activity. This region displays critical cis-acting elements including those for Sp1, the STAT1 and three binding c-sis-inducible elements (SIE), AP2 and NFkB. Further studies on this 813 bp BRK promoter sequence disclosed two specific regions; -93 to -76 bp proposed as the minimal promoter region and the distal -702 to -655 bp region determined to contain the two cis-acting elements needed for binding to NFkB and Sp1 (Kang *et al.*, 2002).

1.1.2.1.2 Protein structure and activation

BRK is a 451 amino acid protein that comprises the prototypical SH3, SH2 and the kinase domains (Fig 1.8). While the SH3 domain is composed primarily of β-sheets (Koo *et al.*, 2002), the SH2 domain consists of an α/β fold and a tyrosine peptide binding surface with two of the α helices arranged opposite to each other on the central β-sheet (Hong *et al.*, 2001; Hong *et al.*, 2004). Though the calculated molecular weight of protein is 52 kDa, BRK is typically found to electrophoretically migrate at around 48 kDa via conventional SDS PAGE techniques.

Studies published by Qiu *et al.* in 2002 and 2004 have described the biochemical characterization of BRK in extensive detail (Qiu and Miller, 2002, 2004). Their studies investigated the regulation of the enzymatic activity of BRK via the intramolecular interactions involving the SH3 and the SH2 domains with their respective intramolecular ligands that reside within the protein. Analogous to Src, while the selective autophosphorylation at Y342 enhanced the catalytic activity via stabilizing the active or the "open" conformation of the enzyme, phosphorylation of Y447 had an opposite effect. Besides Y342, other tyrosine residues that are phosphorylated on BRK include Y13, Y61,

Y66, Y114 and Y351 and may affect effect enzyme activity (Qiu and Miller, 2002) (Fig 1.8). A mutation at K219 renders BRK "kinase-dead" since this lysine is required for ATP-binding. Likewise, a mutation at the autophosphorylation site Y342 rendered the kinase catalytically inactive. However, mutating the Carboxy terminal Y447 has a pronounced effect on enhancing the activity of the kinase via abrogating the catalytically inactivating intra-molecular interaction between the SH2 domain and the peptide containing the tyrosine (Qiu and Miller, 2002, 2004).

Other critical residues involved in such an "autoinhibitory" interaction include the three prolines residues P175, 177 and 179 residing in the N-terminal side of the SH2-kinase linker region that bind to the SH3 domain and W44 that lies within the SH3 domain and initiates contact with the linker region (Kim *et al.*, 2007; Ko *et al.*, 2009; Qiu and Miller, 2002). These residues preserve the autoinhibitory conformation of BRK, and mutating these to alanine result in increased enzymatic activation of BRK (Qiu and Miller, 2002). Specifically, the SH3 domain amino acids reported to be involved in such autoinhibitory interactions with the linker region are R22, W44, N65 and Y66, which upon mutation result in elevated catalytic activity of BRK (Ko *et al.*, 2009) (Fig 1.8). Deleting the SH3 and the SH2 domains individually also results in a higher kinase activity of BRK as demonstrated via autophosphorylation status as well as phosphorylation of the physiological substrate of BRK, Sam68. This suggests the existence of an autoinhibited state of BRK owing to intra-molecular associations, that when relieved, result in an upregulated kinase activity (Qiu and Miller, 2004). W260 in Src family member, Hck sustains a catalytically inhibited state (LaFevre-Bernt *et al.*, 1998). However, in stark contrast to W260 of Src family member Hck, the analogous W184 in BRK plays a pivotal role in the stabilization of the active conformation of the kinase domain. This suggests that unlike Src, the conserved tryptophan in BRK interacts in a different manner with the kinase domain (Kim and Lee, 2005; Qiu and Miller, 2004).

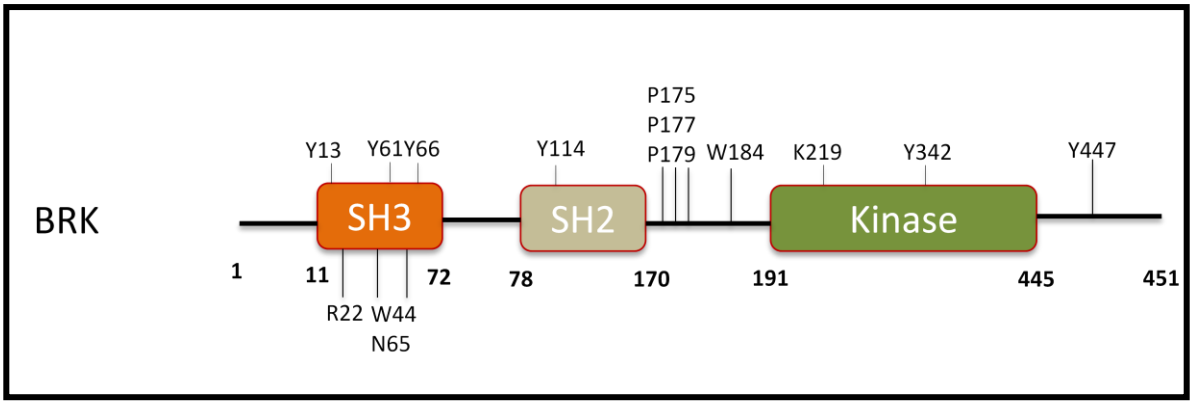

FIGURE 1.8: **Schematic representation of BRK**. Schematic diagram of BRK depicting its various functional domains; the SH3, SH2 and the kinase domains as well as the critical residues involved in the regulation of tyrosine kinase activity. Shown here are the ATP-contacting K219 and the primary autophosphorylation site, Y342, as well as other tyrosine residues known to be autophosphorylated in BRK, namely, Y13, Y61, Y66 and Y114. The residues contributing to autoinhibition of catalytic activity in BRK are Y447 and the SH2-kinase linker residues P175, P177 and P179 which are known to establish intramolecular interactions with the SH2 domain and residues R22, W44 and N65 within the SH3 domain, respectively.

1.1.2.1.3 Expression profile in breast cancer

In Normal cells:

As early as 1995, Vasioukhin and colleagues described the expression of the BRK ortholog, Sik, to be prevalent in the epithelia of the murine skin and the alimentary canal and its expression was found to be exclusive to the renewing epithelial cells in these regions (Vasioukhin *et al.*, 1995). Later studies aimed at delineating tissue-specific BRK expression also confirmed its expression in the gastrointestinal tract which, upon exposure to ionizing radiation, exhibited a marked increase in not only the villus epithelial cells, but also in the crypt compartment of the small intestine (Haegebarth *et al.*, 2009). Specifically, BRK expression was seen in the gastrointestinal tract epithelial cells undergoing terminal differentiation (Llor *et al.*, 1999). Furthermore, BRK was notably expressed in the epithelial lining of the colon as well as that of the prostate, accompanied by a dominant nuclear expression in the latter case (Derry *et al.*, 2003; Llor *et al.*, 1999). Other reports classify BRK expression to normal activated T-cells as well as the cutaenous T-cell lymphomas and transformed T and B-cell populations, characterized by a consistent nuclear localization pattern (Kasprzycka *et al.*, 2006). The expression of BRK has also been documented in

keratinocytes where it exerts a regulatory role in calcium-induced keratinocyte differentiation process (Tupper *et al.*, 2011; Vasioukhin and Tyner, 1997; Wang *et al.*, 2005).

With such a diverse expression profile in normal cells and tissues, it is interesting that BRK is not expressed in the normal mammary tissues or fibroadenomas (benign breast lesion), while persistently overexpressed in the majority of breast carcinomas in humans (Barker *et al.*, 1997; Llor *et al.*, 1999).

In Breast carcinoma

Published reports have shown that BRK, while being low or completely absent from normal mammary tissues, is overexpressed in nearly 86% of breast carcinomas (Aubele *et al.*, 2007; Barker *et al.*, 1997; Ostrander *et al.*, 2007). In a study aimed at determining the cause of BRK overexpression from a panel of 426 invasive breast carcinomas, it was discovered that BRK gene copy number is increased in 45% of such breast tumors with greater than two signals obtained per nucleus via FISH analyses. Furthermore, while gene amplification was evidenced in only 15% of these tumors, polysomy of chromosome 20 was seen in nearly 30% of the same. Nonetheless, a clear statistical and quantitative association between the BRK gene and its corresponding mRNA or protein could not be drawn (Aubele *et al.*, 2009).

1.1.2.1.4 BRK activation in breast cancer

1.1.2.1.4.1 Activation

Besides the ability of BRK to activate via autophosphorylation (Castro and Lange, 2010), there are a number of ligands that can trigger the kinase's activation. This is primarily due to the fact that BRK is involved in several signalling pathways activated by a plethora of ligands such as EGF, HGF, IGF etc. that bind to their respective cell surface receptors, thereby initiating the activation of several downstream targets, one of which is also BRK. For instance, compared to the control, BRK was shown to enhance the growth properties of the MCF10A cell line in response to EGF stimulation (Kamalati *et al.*, 1996). Additionally, in another study that identified IRS-4 as its binding partner, IGF stimulation was shown to enhance BRK activation in the MCF7 breast cancer cell line (Qiu *et al.*, 2005). Furthermore, an activator of the Met receptor, HGF (Hepatocyte Growth Factor) stimulation was also shown to increase BRK kinase activity, by over two folds, in the MDA-MB-231 and T47D breast cancer cell lines (Castro and Lange, 2010). Since the signalling pathways activated by

these ligands tend to promote cell proliferation and migration, it is not surprising that activation of BRK downstream in the signalling pathway mechanistically drives oncogenesis.

1.1.2.1.4.2 Negative regulation of activity

In a recent study, SOCS3 (Suppressor of cytokine signalling 3), was shown to inhibit BRK activation through its association with BRK via its SH2 domain. SOCS3 primarily augments the degradation of BRK, an event mediated mainly by its kinase inhibitory region (KIR) (Gao *et al.*, 2012).

Overexpression of Sam68 (the Src-Associated substrate in Mitosis of 68 kDa) in rat astrocytes was shown to inhibit BRK induced cell cycle progression (Lukong *et al.*, 2005). This hinted towards a negative-regulatory role of Sam68 towards BRK and is suggestive of a mechanism by which BRK activity might be modulated in cells with elevated levels of the Sam68 protein.

1.1.2.1.5 Substrates/binding partners

The number of BRK binding partners highlights the potential of the kinase in regulating a diverse array of signalling pathways *in vivo*. The association of BRK with all four members of the ErbB family (ErbB1/2/3/4) has been shown to regulate mammary tumor progression and growth through stimulation via such growth factors as EGF and Heregulin (Kamalati *et al.*, 2000; Kamalati *et al.*, 1996; Ostrander *et al.*, 2007; Xiang *et al.*, 2008). Some of the early physiological substrates of BRK namely, Sam68, Slm1, Slm2 as well as PSF (Polypyrimidine tract-binding protein associated splicing factor) are RNA binding proteins which show reduced RNA binding following phosphorylation by BRK (Derry *et al.*, 2000b; Haegebarth *et al.*, 2004). BRK also phosphorylates signal transducing adapter family member 2 (abbreviated as STAP-2 and also known as BKS) and indirectly affects cell proliferation and differentiation via the subsequent STAP-2 mediated phosphorylation and activation of STAT3 and STAT5 (Ikeda *et al.*, 2009; Ikeda *et al.*, 2010; Mitchell *et al.*, 2000).

The association of Insulin-like growth factor receptor 1R (IGF-1R) with BRK also holds significance since the former is expressed in many breast cancer subtypes and is linked to poor prognoses (Irie *et al.*, 2010). Regulation of IGF-1R expression and activity by BRK modulates anchorage-independent survival of breast and ovarian cancer cells via the suppression of caspase-mediated apoptosis (Irie *et al.*, 2010). Furthermore, insulin receptor substrate-4 (IRS-4) has also been established as a BRK binding partner and the association

with IRS-4 as well as BRK activity was shown to be enhanced upon IGF-1 stimulation in MCF-7 breast cancer cells (Qiu *et al.*, 2005). ARAP1 (Arf-GAP, Rho-GAP, ankyrin repeat, and pleckstrin homology (PH) domain-containing protein 1) was another BRK substrate identified recently which, upon phosphorylation by BRK, was shown to down-regulate EGFR levels in the BT-474 and T47D breast cancer cell lines (Kang *et al.*, 2010).

Moreover, BRK was also found to complex with AKT in the T47D breast cancer cell line upon EGF stimulation (Zhang *et al.*, 2005). In another study, BRK was shown to directly phosphorylate AKT on tyrosines 315 and 326, resulting in an unequivocal enhancement of cellular proliferation in response to EGF stimulation in the mouse embryonic cell line, SYF (Zheng *et al.*, 2010).

Other associating partners of BRK have been found in the Adamalysins family of transmembrane proteins, abbreviated as ADAM (Zhong *et al.*, 2008). Of the four alternatively spliced products of the ADAM15 gene, BRK has been characterized to bind strongly to ADAM15A and ADAM15B with modest binding to ADAM15C. While the expression of the ADAM15 protein varies significantly amongst breast cancer cells as compared to normal mammary cells, higher levels of its two alternatively spliced forms, ADAM15A and ADAM15B, have been correlated with poor relapse-free survival in node-negative patients as opposed to elevated ADAM15C levels correlating with a better relapse-free survival in node-positive patients (Zhong *et al.*, 2008).

An important component of the Wnt signalling pathway, β-catenin, was also characterized as a bona fide substrate of BRK. The Wnt/β-catenin signalling plays a prominent regulatory role in the growth of several types of cancers (Beildeck *et al.*, 2010; Robinson *et al.*, 2008). The phosphorylation of β-catenin by BRKcauses a significant drop in the transcriptional activity of the β-catenin (Palka-Hamblin *et al.*, 2010). Furthermore, as studied in the SW620 human colorectal cancer cell line and mouse intestines, whereas nuclear-targetted BRK was found to negatively regulate β-catenin/T-cell Factor (TCF) mediated transcription, membrane targeted BRK displayed an opposite effect (Palka-Hamblin *et al.*, 2010). Interestingly, even the alternatively spliced variant of BRK, ALT-BRK, has been shown to bind to β-catenin, but more importantly, has been implicated in enhancing BRK-mediated transcriptional repression of β-catenin (Brauer *et al.*, 2011).

Very recently, a study ascribed the role of BRK to the formation of peripheral adhesion complexes via phosphorylation of p130CAS (CRK associated substrate), a protein

that condenses at focal adhesions within cells (Zheng *et al.*, 2012a). p130CAS was validated as a direct substrate of BRK and phosphorylation of the same by the membrane-targeted form of the kinase, was shown to induce cell migration through ERK5 activation (Zheng *et al.*, 2012a). Reminiscent of the role of BRK in cell migration, phosphorylation of its other direct substrate, paxillin, upon EGF stimulation was also shown to regulate a similar metastatic invasion in cancer cells via GTPase Rac1 activation (Chen *et al.*, 2004).

In a separate publication, Focal adhesion kinase (FAK) was discovered as a bonafide substrate of BRK via mass spectrometry analysis of BRK-phosphorylated targets in the human SW620 colon cancer cell line (Zheng *et al.*, 2012b). BRK was shown to activate FAK via direct phosphorylation in the murine SYF cell line, thereby protecting the cells from contact inhibition-mediated growth suppression. Furthermore, while knock-down of BRK down-regulated FAK activation, exogenous restoration of the latter conferred resistance to anoikis (programmed cell-death resulting from the detachment of anchorage-dependent cells from the extra-cellular matrix) in the PC3 prostate cancer cell line, demonstrating the significance of BRK in suppressing cellular anoikis (Zheng *et al.*, 2012b).

1.1.2.1.6 Cellular roles: proliferation and migration

1.1.2.1.6.1 Cell proliferation

That BRK is overexpressed in the majority of breast carcinomas is a good reason to investigate its role as an oncoprotein. Indeed, BRK has been well characterized for its prominent role in inducing cell proliferation and regulating cell cycle progression in breast carcinomas, substantiating its function as an oncoprotein (Brauer and Tyner, 2010). In one of the early published reports highlighting the involvement of BRK in cell growth, it was shown that the BRK-transfected human mammary cell lines, Hb4a and MCF10A, exhibited an increase in their growth properties following EGF stimulation (Kamalati *et al.*, 1996). The same study also described a significant transforming potential of BRK since the Hb4a and mouse fibroblast, NIH3T3, cell lines both demonstrated the ability to grow in an anchorage-independent manner following stable transfection with BRK. Relevant to these results, in a very recent study, it was demonstrated that BRK directly phosphorylates tyrosine 845 on EGFR and confers resistance against cetuximab (an antibody against EGFR) (Li *et al.*, 2012). This has important implications since EGFR activation via ligand stimulation leads to cell proliferation (Schlessinger and Ullrich, 1992; Tajeddine and Gailly, 2012; Vieira *et al.*,

1996). In the non-tumorigenic cell line MCF10A, BRK was also shown to associate with ErbB2, a receptor tyrosine kinase belonging to the EGFR family, and induce cell proliferation via the activation of the Ras/MAPK pathway (Xiang *et al.*, 2008).

Another study revealed that phosphorylation of P190-RhoGAP-A (p190) by BRK elicits an increase in cellular proliferation in the MCF7 breast cancer cell line by inducing the formation of a complex between p190 and p120RhoGAP (p120). This results in the stimulation and attenuation of p190 and p120 functions, respectively (Shen *et al.*, 2008). Other researchers have also described a direct role of BRK towards cell proliferation. BRK has been shown to directly phosphorylate AKT on tyrosines 315 and 326, thereby activating the serine/threonine protein kinase and potentiating cell proliferation in response to growth factor stimulation (Zheng *et al.*, 2010). Under conditions of EGF and heregulin stimulation, breast cancer cell lines such as T47D, MCF7 and MCF10A showed enhanced endogenous BRK autophosphorylation resulting in cell proliferation via the activation of Rac GTPase, extracellular signal regulated kinase 5 (ERK5) and p38 mitogen-activated protein kinase (MAPK) (Ostrander *et al.*, 2007). The involvement of canonical adaptor proteins such as that of STAP-2 in BRK-induced cell proliferation has also been investigated. Robust tyrosine phosphorylation of STAP-2 by BRK was shown to initiate the activation of STAT3 as well as STAT5b and promote increased transcriptional activity and proliferation in breast cancers (Ikeda *et al.*, 2011; Ikeda *et al.*, 2010). Relevant to its role in enhancing cell proliferation, it was also discovered that the suppression of BRK in breast carcinoma induces autophagic cell death while the ectopic expression of the same was found to enhance cell survival (Harvey *et al.*, 2009).

The oncogenic function of BRK was attributed to its cytosolic/membrane localization, while an antagonistic (tumor suppressive) role was ascribed to its nuclear containment (Ie Kim and Lee, 2009). For instance, while incorporation of a myristoylation signal leading to membrane localization caused BRK to promote cell proliferation and migration via enhancing β-catenin transcription and Wnt/β-catenin signalling, a nuclear distribution of the kinase inhibited such functions (Palka-Hamblin *et al.*, 2010). However, the nuclear distribution of BRK may not necessarily correlate with anti-tumorigenic effects. For instance, the co-localization of BRK with Sam68 in Sam68-like nuclear bodies (SNBs) within the nucleus results in reduced RNA binding properties of Sam68, a functional characteristic that is believed to be a mechanism by which BRK contributes to oncogenecity (Derry *et al.*, 2000b; Lukong *et al.*, 2005). Furthermore, cellular re-distribution may not

always be limited to BRK as an outcome of target protein phosphorylation, but also to the target protein itself. For instance, BRK was shown to phosphorylate and yield a cytoplasmic re-localization of PSF from the nucleus and which subsequently lead to cell cycle arrest (Lukong *et al.*, 2009).

Other functions of BRK have been noted in DNA damage/apoptosis. For instance, BRK overexpression was found to sensitize rat fibroblast cells to conditions of stress (serum starvation) and apoptotic stimuli (UV radiation) (Haegebarth *et al.*, 2005). However, in a very recent study BRK knockdown displayed enhanced apoptosis upon UV radiation in colon tumor cells (Gierut *et al.*, 2012). Interestingly, there are also reports of a tumor suppressive role of BRK in esophageal carcinoma with BRK expression determined to be downregulated in tumorigenic tissues derived from the larynx and esophagus compared to matched adjacent normal tissues (Liu *et al.*, 2013; Ma *et al.*, 2012).

1.1.2.1.6.2 Cell migration

Besides positively regulating cellular proliferation in breast carcinomas, BRK can also influence the cells' migratory properties. Upon EGF stimulation, BRK is activated by EGFR resulting in the phosphorylation of paxillin specifically at tyrosines 31 and 118. The cellular ramifications of such a post-translational modification on paxillin involve the binding of CRKII to paxillin resulting in the activation of Rac and the formation of lamellipodia leading to cell motility/migration (Chen *et al.*, 2004). Other factors causing BRK-mediated cell migration involve the stimulation of cells via such ligands as Heregulin, EGF and HGF (Castro and Lange, 2010; Chen *et al.*, 2004; Ostrander *et al.*, 2007). Phosphorylation of p190RhoGAP by BRK also results in an enhanced chemotactic migration of cells as studied in mouse embryonic fibroblasts (Shen *et al.*, 2008). Furthermore, its physiological substrate, KAP3A (kinesin superfamily-associated protein 3) was also shown to necessitate BRK-induced cell migration in the BT20 breast cancer cell line (Lukong and Richard, 2008).

1.1.2.1.7 Mouse models

The role of BRK in tumor formation has been investigated through comprehensive studies on mouse models. Such animal models have proved invaluable towards deciphering the function of BRK in the context of tumorigenesis. Intriguingly, BRK null (knockout) mice displayed characteristics of enhanced cellular proliferation and growth, in the enterocytes of their small

intestines as compared to their BRK-positive counterparts (Haegebarth *et al.*, 2006). Additionally, consistent with its role in promoting cell differentiation, intestinal samples derived from such BRK knockout mice also showed delayed expression of the differentiation marker I-FABP (Intestinal fatty acid binding protein) (Haegebarth *et al.*, 2006). These results are however indicative of a growth-inhibitory role of BRK, consistent with this group's prior data.

In a more recent publication, *in vitro* results demonstrating the ability of BRK to suppress cellular anoikis was confirmed in *in vivo* experiments using xenograft mouse models. Sub-cutaenous injection of SYF cells over-expressing membrane-targeted BRK (designated Palm-BRK-YF), into nude mice, led to the formation of tumors 4 weeks post-injection (Zheng *et al.*, 2012b). This, therefore, validates the novel means by which BRK induces oncogeneis in transformed cells via suppressing anoikis.

Transgenic mouse models exhibiting an inducible BRK targeted expression in the mammary gland have been developed and characterized for tumorigenesis. The effects of targeted wild type BRK expression in mouse mammary glands was studied on involution and tumor formation as well as the associated signalling pathway through which such oncogenic functions are manifested. It was found that BRK overexpression resulted in delayed involution of the mammary glands and early tumor growth via the p38 MAPK cascade activation (Lofgren *et al.*, 2011). In a subsequent study which examined the oncogenic function of a constitutively active variant of BRK, it was reported that the stable expression of Y447F (constitutively active variant) BRK in the MDA-MB-231 breast cancer cells resulted in a higher rate of cell proliferation as well as migration, in comparison to wild-type BRK (Miah *et al.*, 2012). Furthermore, *in vivo* results from xenograft mouse models also indicated the need for the full activation of BRK to promote a malignant phenotype (Miah *et al.*, 2012).

1.1.2.2 Fyn-related Kinase (FRK)

1.1.2.2.1 Gene discovery and organization

Another member of the FRK/PTK6 family kinases, FRK, is also known as PTK5. As early as 1993, attempts to identify novel tyrosine kinases in the human breast cancer cell line PEI600 as well as in primary breast carcinoma, led to the discovery of FRK (then designated as TK1, for tyrosine kinase 1) (Cance *et al.*, 1993). Endeavours to further characterize this

505 amino acid protein also brought about its new designation, RAK, which is a Russian word for "cancer" (Cance *et al.*, 1994). The protein displayed 49% amino acid sequence similarity to the human proto-oncogene FYN kinase and was therefore termed Fyn-Related Kinase (FRK) (Lee *et al.*, 1994). Besides being expressed in breast carcinomas, such as the breast cancer cell line BT20 and the LS180 colon cancer cell line, FRK was also found to be expressed in epithelial cells of the human kidney and liver while being absent from the cells of mesenchymal origin such as that of the skeletal muscle (Cance *et al.*, 1994). FRK maps to chromosome 6q21-q22.3 in humans (Brauer and Tyner, 2009) within a region that is atypically destabilized by loss of heterozygosity (LOH) in nearly 48% of breast tumors (Sheng *et al.*, 1996) as well as malignant melanomas (Becher *et al.*, 1983a; Becher *et al.*, 1983b).

The identification and expression profile of the murine orthologs of FRK have also been documented and account for its varied nomenclature. Designated IYK (Intestinal tyrosine kinase) or BSK (beta-cell Src-homology kinase), murine FRK was cloned from the mouse pancreatic cell line, RINm5F (Oberg-Welsh and Welsh, 1995) as well as the mouse mammary gland (Thuveson *et al.*, 1995). Northern blot analysis and RT-PCR techniques confirmed the expression of the genes in the kidney, liver, lung as well as the intestines (Oberg-Welsh and Welsh, 1995; Thuveson *et al.*, 1995). Another FRK homologue, named GTK (Gut tyrosine kinase), was cloned from the small intestine in rats and also displayed a similar expression pattern (Sunitha and Avigan, 1994, 1996). However, the focus of this section shall rest primarily with the human form of the kinase and referred to as FRK for simplicity.

As mentioned in the previous sections, like SFK's as well as BRK and SRMS, FRK also possesses all three functional domains, i.e., the SH3, the SH2 and the kinase domain. A unique feature in FRK is the presence of a nuclear localization signal (NLS) within the SH2 domain. Such a bipartite motif, organized in two clusters of basic amino acids and separated by a spacer of nine amino acids, has the characteristic sequence: KRLDEGGFFLTRRR. The motif was shown to dictate nuclear localization of endogenous FRK in the monkey kidney cell line, COS7 (Cance *et al.*, 1994). Other reports indicated that in the lactating normal human breast tissues, the proliferating epithelial cells display a predominant cytoplasmic localization of FRK, while a more prominent nuclear distribution was noted in the post-menopausal epithelial cells (Kaplan *et al.*, 1987). In a study aimed at investigating the biological implications of ectopic IYK expression in the murine fibroblast

cell line NIH3T3, it was shown that a C-terminal mutation, Y497F, conferred upon the protein the ability to re-localize to the nucleus since the wild type protein was predominant only in the cytosolic sub-cellular compartment (Oberg-Welsh *et al.*, 1998). In a subsequent report, these findings were corroborated in another experimental cell line, the murine pancreatic RINm5F cell line (Anneren and Welsh, 2000). In yet another study, the deletion of the NLS-containing SH2 domain was shown to cause a diffuse cytoplasmic localization of FRK as opposed to the perinuclear distribution of the ectopically expressed wild-type protein (Meyer *et al.*, 2003). Whereas deletion of the SH3 domain did not impact sub-cellular localization, mutation of the ATP-contacting lysine (K262) residue lying within the kinase domain yielded a punctate-perinuclear distribution of FRK in the BT474 breast cancer cell line (Meyer *et al.*, 2003). However, even as FRK possesses a nuclear localization motif, it lacks a plasma membrane-directing myristoylation signal, a feature found in its murine homologue (Serfas and Tyner, 2003).

1.1.2.2.2 Protein structure and activation

The 1.9 kb cDNA of FRK encodes a 505 amino acid protein with a molecular weight of roughly 54 kDa. The orientations of the functional domains, namely, SH3, SH2 and the kinase, remain conserved with BRK and SRMS and these domains do not deviate from their respective modes of action, although ligand specificity may vary. In comparison to BRK, FRK has been only modestly characterized for its enzymatic activity. The earliest reports of intrinsic FRK kinase activity came from a study that also identified the protein. Through an *in vitro* kinase assay using ^{32}P, performed on immunoprecipitated FRK from the BT20 breast cancer cell line, it was shown that the wild-type kinase demonstrated the capability of autophosphorylation and also immunospecificity towards an anti-phosphotyrosine antibody, thereby presenting itself as a functional tyrosine kinase (Cance *et al.*, 1994). The study had identified Y387 as the putative autophosphorylation site in FRK (Cance *et al.*, 1994). Experimental corroborations were illustrated in another study aimed at investigating the enzymatic ramification of point-mutations made in the carboxy-terminus of FRK's highly homologous relative, IYK. Analogous to c-Src, IYK possesses a tyrosine- Y497 in addition to another Y504, in its C-terminal region (Fig 1.9). Like Src and BRK, the presence of a spatially conserved tyrosine (Y504) in the C-terminal region of IYK is expected to exert a regulatory effect on its enzymatic activity, via binding to the SH2 domain upon phosphorylation. The study supported this hypothesis, noting that while Y504F (single) and

Y504F/Y497F (double) mutations on IYK resulted in increased autophosphorylation, Y497F alone did not appreciably exhibit such an increase in intrinsic kinase activity, in comparison to the wild-type protein (Anneren and Welsh, 2000; Oberg-Welsh *et al.*, 1998). A subsequent report characterized the catalytic activity of FRK. The study published that while the deletion of the SH2 and SH3 domains had no impact on the autophosphorylation of FRK, mutation of a single lysine (K262R) within the kinase domain rendered the protein catalytically inactive (Meyer *et al.*, 2003).

CSK was originally identified to phosphorylate members of the Src family kinases and down-regulate their enzymatic function (Nada *et al.*, 1991; Okada *et al.*, 1991; Okada and Nakagawa, 1989; Partanen *et al.*, 1991; Sabe *et al.*, 1992). Cance and colleagues remain the only group to date to highlight CSK (c-Src kinase) as a potential regulator of FRK kinase activity (Cance *et al.*, 1994). Through the generation of four disparate peptides matching the carboxy terminus tail of FRK, but individually mutated for each of the four tyrosines Y450, Y463, Y490 and Y497, it was determined in an *in vitro* kinase reaction that CSK demonstrates a greater phosphotransfer affinity towards Y497 over the other tyrosines and that this could potentially confer a regulatory effect on the catalytic activity of FRK (Cance *et al.*, 1994).

1.1.2.2.3 Substrates/binding partners

To date, PTEN and EGFR remain the sole authenticated substrates of FRK, even as there have been reports of several binding partners. Furthermore, minimal information regarding SH3/SH2 interactions of FRK with its binding partners, hinders the understanding of the cellular functions of FRK. Nonetheless, the FRK SH2 domain has been recently characterized for its ligand specificity. Through a proteomics study encompassing binding assays coupled with mass spectrometry analyses, it was shown that for a given phosphotyrosine-containing ligand, the FRK SH2 domain prefers a glutamic acid or cysteine residue at the P+1 position (where "P" refers to the phosphotyrosine residue) while at the P+3 position, an isoleucine is favoured in addition to a cysteine (Zhao *et al.*, 2013) (Table 1.1). Preceding the phosphotyrosine residue, a methionine or a tyrosine residue is favoured at the P-1 position on the ligand. While there exists a certain degree of overlap in the positional variation in amino acids between FRK, SRMS and BRK, certain residues as described above define the characteristic preference of the FRK SH2 domain in establishing distinct interactions with specific ligands/binding partners (Zhao *et al.*, 2013).

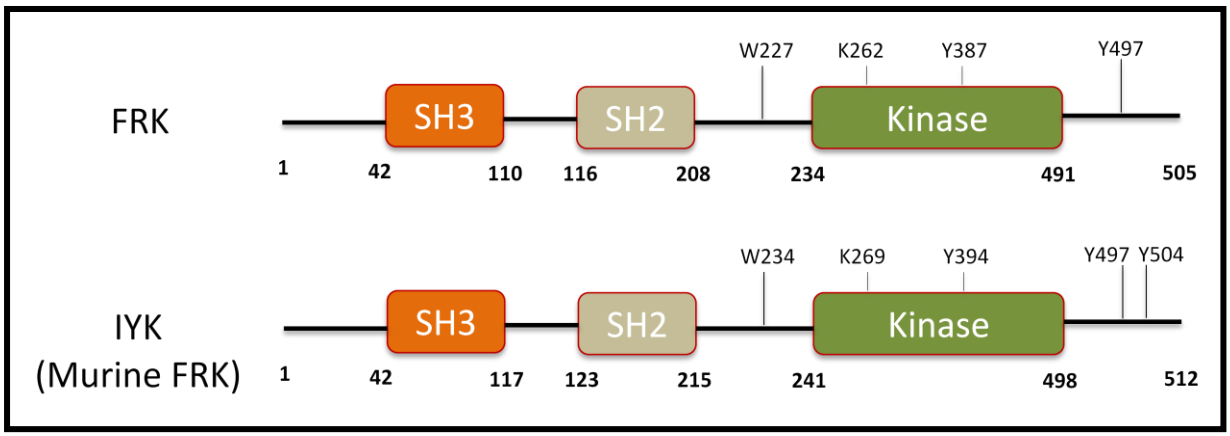

FIGURE 1.9: **Schematic representation of FRK**. Schematic diagram of the human and mouse variants of FRK depicting the various functional domains; the SH3, SH2 and the kinase domains as well as the critical residues involved in the regulation of tyrosine kinase activity, namely the ATP contacting K262 (K269 in IYK), the autophosphorylation site, Y387 (Y394 in IYK) and an autoinhibitory tyrosine residue, Y497 (Y504 and Y497 in IYK), within the C-terminus of FRK. Also shown is W227 (conserved with W234 in IYK), situated within the SH2-Kinase linker segment, which is highly conserved amongst Src family kinases as well as BRK and SRMS and has been reported to be involved in the regulation of the active/inactive conformation of certain tyrosine kinases.

Interestingly, with FRK, the canonical SH3 and SH2 domains were recently shown to function in a manner distinct from BRK as well as the Src family. While individual deletion of the FRK SH3 or the SH2 domains did not influence the interactions of the tyrosine kinase with EGFR, composite deletion of both domains together resulted in diminished associations with EGFR, thus suggesting a cooperative mechanism of functioning by both, the SH3 and SH2 domains (Jin and Craven, 2013).

The human retinoblastoma protein, abbreviated pRb, was the first documented binding partner of FRK and primarily accounted for the association of a potential tumor suppressor function to the non-receptor tyrosine kinase. pRb, a tumor suppressor (Benedict *et al.*, 1983), possesses two protein binding pockets: the larger A/B pocket, which has been shown to interact with proteins such as E2F and cyclin D (Weinberg, 1995) and a smaller C pocket reported to interact with the c-Abl tyrosine kinase (Welch and Wang, 1993). It is through the A/B pocket that FRK associates with pRb (Craven *et al.*, 1995). However, the biological significance of such a binding interaction is not clear with only minor speculations

of FRK's greater proximity to other cellular substrates via pRb association being noted (Yim *et al.*, 2009b).

PTEN, another tumor suppressor, was reported as a bonafide FRK substrate in a study that not only characterized the mechanistic association between the two proteins but also decoded a functional relevance of the same (Yim *et al.*, 2009a). FRK was shown to phosphorylate PTEN specifically on Y336, upon binding, requiring the FRK SH3 and the PTEN C2 domain. Thus, via phosphorylation, FRK was also shown to stabilize PTEN by protecting PTEN from the cell's degradative machinery involving poly-ubiquitination (Yim *et al.*, 2009a).

In a recent report, it was shown that FRK enhances EGFR phosphorylation at Y1173 while suppressing its phosphorylation at Y1068 (Jin and Craven, 2013). Such site-specific and direct phosphorylation of EGFR by FRK was depicted to result in the internalization of the cell surface-bound EGFR (Jin and Craven, 2013).

Other binding partners reported for FRK include CDC2 and Cdk4 (Pendergast, 1996). While evidence in support of this information remains absent from the literature, it was nonetheless only mentioned in a study on nuclear tyrosine kinases that FRK associates specifically with CDC2 and Cdk4 and not with Cdk5, Cdk6 or Cdk7 (Pendergast, 1996). Furthermore, the functional significance of such interactions also remains unexplored.

In another study evaluating the physiological significance of GTK (an FRK homologue), its exogenous expression was demonstrated to correlate with both the quantitative enhancement as well as the phosphorylation of several protein targets to induce neurite outgrowth in the mouse PC12 cell line, upon nerve growth factor stimulation (Anneren *et al.*, 2000). These include proteins such as Focal Adhesion Kinase (FAK), SHB (an adapter protein that stands for Src homology 2 protein of Beta cells) and TrkA (a neuronal receptor). However, their molecular interactions with GTK were not determined in the same report. A subsequent article further reported on the functioning of FRK upstream of SHB towards enhancing PC12 cell differentiation and beta-cell proliferation (Anneren *et al.*, 2003).

1.1.2.2.4 Expression profile in breast cancer

In Normal cells:

While varying mRNA levels were detected, via Northern blot analysis, in the epithelial tissues derived from the adult human liver, muscle, kidneys and pancreas, expression of the

corresponding protein was observed only in the kidney and liver (Cance *et al.*, 1994). A later study suggested that while significant FRK mRNA expression was observed in the normal tissues derived from the lungs, a minimal expression was also seen in liver tissues (Chen *et al.*, 2013). Moreover, FRK expression was also reported in the normal human breast epithelial tissues (Berclaz *et al.*, 2000).

In Breast carcinoma:

Unlike BRK, FRK expression in the multitude of breast carcinomas remains poorly characterized. The only investigation on its expression pattern in breast cancer comes from the group which also identified the protein. The first reports of FRK expression came from the human breast cancer cell lines, SKBR3, BT-20 and PEI600 including primary breast tumors (Cance *et al.*, 1994; Cance *et al.*, 1993; Craven *et al.*, 1995). Other reports from analyses on a panel of 21 invasive breast carcinoma samples revealed either a complete loss or significantly diminished expression of FRK compared to normal breast tissues (Berclaz *et al.*, 2000). Subsequent studies also reported on the presence of FRK transcripts in the MDA-MB-468 and MCF-10A breast-derived cell lines (Yim *et al.*, 2009a; Yim *et al.*, 2009b).

Additionally, FRK expression was also illustrated in the colon cancer cell lines; LS180 and Colo205 as well as the hematopoietic tumor cell lines, K562 and HL60 besides being detectable in the human lymphoma cell line U937 and a rhabdomyosarcoma (RD) cell line (Cance *et al.*, 1994). FRK has also been characterized as an aberrantly overexpressed protein in hepatocellular carcinoma (Chen *et al.*, 2013).

1.1.2.2.5 FRK activation in carcinomas

Cance and colleagues showed via immunoprecipitation that in the metastatic liver tumors, FRK is highly autophosphorylated (Cance *et al.*, 1994). This not only correlated with the expression levels of the protein, but also contradicted the condition observed in the normal colonic tissues and those obtained from primary tumors where a limited expression of FRK paralleled a low autophosphorylation status (Cance *et al.*, 1994). Nonetheless, the underlying mechanism governing such activation of the kinase is yet to be explored.

1.1.2.2.6 Cellular roles- proliferation and migration

Even in the absence of adequate data on FRK, compelling evidence is available to support its status as a potential tumor suppressor. Oberg-Welsh and colleagues reported that ectopic

expression of its constitutively active mutant, harbouring a double mutation in its C-terminus region (Y497F/Y504F), resulted in an impaired/reduced cellular proliferative rate in the NIH3T3 cell line in comparison with cells transfected with either the wild type or the kinase-dead mutant (K269A) (Oberg-Welsh *et al.*, 1998). FRK was shown to exhibit such a function via regulating cell cycle progression as evidenced from cell cycle analysis as well as quantitative measurement of exogenous thymidine (methyl-^{3}H) incorporation into the DNA of actively dividing cells (Oberg-Welsh *et al.*, 1998). These findings suggest the non-receptor tyrosine kinase as a negative regulator of cell proliferation. As well, in their subsequent publication, the group demonstrated that the overexpressed IYK mutants, Y504F and Y497F/504F also negatively impacted cellular proliferation rates in the rat pancreatic cell line, RINm5F by promoting cell cycle arrest in the G1 phase besides modulating glucagon synthesis via inducing an increase in the mRNA levels of IYK (Anneren and Welsh, 2000). Furthermore, such ectopic expression of IYK was also found to sensitize the cells to cytokine induced cell death (Anneren and Welsh, 2000). However, an interesting feature noted in both studies was the association between the nuclear distribution of IYK and its inhibitory effect on cell proliferation.

Another early study that also identified pRb as its first binding partner in the SKBR3 breast cancer cell line, provided possible biological basis for the tumor suppressive function of FRK (Craven *et al.*, 1995). The association between active pRb and FRK in the G1 and S phases of cell cycle and not in the Mitotic phase, together with the knowledge of sporadic mutations in the A/B pocket (site for FRK binding) of pRb in several cancers, suggested a potential mechanism supporting the tumor suppressive role of FRK (Craven *et al.*, 1995).

Another major study revealed that while the sub-cellular distribution of FRK in the normal human breast tissues was hormone-dependent; the expression of the kinase was lost in malignant breast carcinomas (Berclaz *et al.*, 2000). Immunohistocytochemical analysis of breast tissue sections obtained at different stages of the menstrual cycle showed a strong cytoplasmic staining of FRK during the more proliferative follicular and luteal phases in contrast to the predominant nuclear distribution of the kinase post-menopause. Furthermore, such dependence on the estrogen-progesterone hormonal status was virtually lost in breast cancer where diminished expression of FRK was seen in the invasive carcinomas (Berclaz *et al.*, 2000). Results from this investigation not only reinforced the significance of the nuclear

localization of the kinase for mediating a growth-inhibitory effect but also partially reasoned the maintenance of proliferative abilities in breast carcinomas.

Direct evidence, in the context of FRK-mediated attenuation of cell proliferation, was also presented by another group which demonstrated that in the BT474 and MCF7 breast carcinoma cell lines, ectopic FRK resulted in growth arrest in the G1 stage of cell cycle and that such an outcome was dependent on its kinase activity since its kinase-inactive mutants exhibited a null effect on cellular proliferation (Meyer *et al.*, 2003). Furthermore, it was also noted that the ability of FRK to down-regulate cell proliferation in these cell lines was independent of pRB association (Meyer *et al.*, 2003).

Negative regulation of cellular proliferation via PTEN stabilization is a concept that was recently put forth (Yim *et al.*, 2009a). The study reported that FRK stabilizes the potent tumor suppressor PTEN by phosphorylating it on Y336, thereby protecting PTEN from ubiquitin-mediated proteasomal degradation. Furthermore, the same study also demonstrated that the *in vitro* knockdown of FRK in the non-tumorigenic MCF10A breast epithelial cell line was sufficient to transform the cells via PTEN degradation, thus ascribing a bonafide tumor-suppressive role to FRK (Yim *et al.*, 2009a).

More recently, in a novel discovery of the signalling pathway associated with FRK functions, Zhou *et al.*, presented evidence for the role of FRK in suppressing glial cell tumorigenesis (Zhou *et al.*, 2012). While FRK mRNA and its associated protein levels were found to be low in the glioma tissues as compared to their non-tumorous brain counterparts, scratch test as well as three-dimensional invasive assays conducted on the U251 and U87 glioma cell lines, demonstrated the ability of ectopic FRK to suppress cell migration and invasion. Furthermore, FRK was shown to potentiate such tumor-suppressive effects via suppressing JNK and consequently, c-Jun phosphorylation (Zhou *et al.*, 2012). Another study supporting the tumor-suppressive role of FRK, described the mechanistic potential of the tyrosine kinase in phosphorylating EGFR at the cell-surface, thus triggering the internalization of the receptor (Jin and Craven, 2013). Since EGFR overexpression is implicated in cellular oncogenesis (Bhargava *et al.*, 2005; Rimawi *et al.*, 2010), FRK-mediated downregulation of the surface-bound receptor population could potentially suppress the propagation of signalling pathways involved in cell proliferation and migration.

In another interesting discovery from the genome of a chronic leukemia patient, a chimeric fusion of the FRK protein with the oncogenic ETV6 transcription factor was reported as a consequence of an unusual in-frame mutagenic union between exon 4 of the

ETV transcript and exon 3 of FRK (Hosoya *et al.*, 2005). The chimerically fused protein was found to be biologically active as evidenced not only by its ability to transform the Baf3 and NIH3T3 murine cell lines in a kinase dependent manner, but also by the observation of tyrosine autophosphorylation within the protein as well as the direct phosphorylation of histone targets H2B and H4. Furthermore, its intrinsic kinase activity together with its inhibited capacity in inducing ETV-mediated transcriptional repression, collectively support the involvement of the chimeric protein in leukemogenesis (Hosoya *et al.*, 2005).

Nonetheless, contrasting a widely speculated cancer-antagonistic role of FRK, there was the recent discovery of the expression and correlation of FRK in hepatocellular carcinoma (HCC) (Chen *et al.*, 2013). In their study, Chen *et al.* reported on the generation of a comprehensive, web-based tumor-associated gene (TAG) database to facilitate the exploitation of cancer gene annotation. The results of the investigation demonstrated that both, the mRNA transcript as well as the protein levels of FRK were more than twice the normal levels in over 50% of HCC tissue samples. Furthermore, an *in vitro* invasive ability of FRK was witnessed in the human liver cell lines, HepG2 and Hep3B as monitored in a matrigel invasion assay since FRK- knockdown HepG2 cells exhibited diminished migration over its FRK-overexpressing counterpart (Chen *et al.*, 2013). Nevertheless, that FRK overexpression correlates with HCC in a manner analogous to BRK and breast cancer is not quite surprising. FRK has been previously documented for its elevated expression in breast carcinomas as well as colon cancers (Armistead and Thorp, 2002; Cance *et al.*, 1994).

Hints towards the involvement of FRK's other homologue, GTK, in cellular differentiation stemmed from the observation of an unusual concentration of the active kinase at the membrane borders of the epithelial cells, lining the murine small intestine. The observation of such *in vivo* localization of the hepatocyte growth factor (HGF)-stimulated enzymatically active kinase, at all stages of cellular maturation, suggested a mode of signalling that could potentially promote gut epithelial cell differentiation in rats (Sunitha and Avigan, 1996). However, another study reported IYK deficient mice (IYK knockout mice) to be generally devoid of phenotypic variations. Specifically, no alterations were observed in the intestinal brush border epithelial morphology, suggesting an expendable need for IYK in intestinal cell differentiation, results that contrast those of Sunitha *et al.* (Sunitha and Avigan, 1996). Yet, overexpressed GTK was also found to enhance FAK, TrkA and SHB phosphorylation via Rap1 activation to promote cellular differentiation in the PC12 murine cell line (Anneren *et*

al., 2000). Therefore, while *in vitro* evidence exists for a positive outcome for FRK in cellular differentiation, *in vivo* results seem elusive and need further investigation.

1.1.2.2.7 Mouse models

Mouse models for FRK/IYK have been described in several studies. Nonetheless, while only a few of them address tumor/cancer biology, the majority of such investigations direct attention towards other functional consequences that appear to be tissue-specific.

In one of the first reports characterizing IYK knockout mice, IYK deficiency yielded no obvious phenotypical deviations in the mice litter (Chandrasekharan *et al.*, 2002). Such mice were viable, healthy and fertile and survived without developing any physiological deficits. However, such loss of IYK expression was compensated by elevated RNA levels of certain Src family kinases (SFK) like c-Src and YES in the colonic and intestinal cells (normal sites of basal IYK expression in normal/wild-type mice), indicative of a compensatory role of SFK's towards IYK. This apart, a significant reduction of over 80% in the circulatory levels of the T3 thyroid hormone was also detected in these mice (Chandrasekharan *et al.*, 2002). However, while being apparently redundant due to the absence of phenotypical changes in these mice, IYK functions need further investigation.

Other IYK-null mice were developed in a series of studies that were focussed on assessing an endocrinological impact of FRK. Even in the absence of observable physiological changes in IYK-null mice, subsequent studies managed to discover subtle deviations from a normal phenotype. For instance, FRK-deficient mice were reported to exhibit a marked reduction in pancreatic cell number in the embryonic and early post-natal stages of their life-cycle (Akerblom *et al.*, 2007). These reports gained momentum from two separate investigations employing GTK-overexpressing transgenic mice. Mice expressing a kinase active (Y504F) mutant of GTK, not only exhibited increased pancreatic β-cell mass and area but such extracted islets also demonstrated a pronounced sensitivity to cytokine-mediated cell death upon exposure to IL-1β and ILF-δ (Anneren and Welsh, 2001). Furthermore, elevated phosphorylated levels of ERK1/2 with a concomitant activation of mitogen activated protein kinase, were hallmarks exclusive to the transgenic pancreatic islets as opposed to the controls (Anneren and Welsh, 2001). In the other study that also examined the ramifications of Src Homology 2 protein of Beta cells (SHB) in rat endocrinology, the GTK overexpressing transgenic litter exhibited increased β-cell proliferation to levels comparable with those of SHB overexpressing mice. As well, an enhanced chemical

sensitivity to the toxin Streptozotocin (STZ) was observed, which potentiated β-cell loss and an impaired glucose tolerance in these mice (Anneren, 2002). Taken together, these results demonstrate a positive role for GTK in β-cell proliferation, yet a concurrent accession in sensitivity to cytokines leading to β-cell loss also indicates an impact of the tyrosine kinase in type-I Diabetes (Anneren, 2002; Anneren and Welsh, 2001, 2002). Consolidating results were also presented in a subsequent study, wherein such transgenic mice demonstrated reduced islet blood flow per islet volume, a condition that accompanied a physiological reduction in the pancreatic capillary lumen diameter as well as a type-II diabetic condition as evidenced from reduced *in vivo* insulin secretion (Anneren *et al.*, 2007).

In the only mouse model generated to date to progress knowledge on FRK's anti-tumorigenic armament, control MCF-10A cells injected into the mice mammary glands, failed to produce tumors, while FRK-knockdown MCF-10A cells led to tumor development in as early as 3 weeks post injection (Yim *et al.*, 2009a). Similarly, mice injected with control MCF7 cells developed tumors in 8 weeks whereas those injected with the FRK-overexpressing cells remained tumor-free. With these experimental results, FRK was deemed a bonafide tumor suppressor (Yim *et al.*, 2009a).

However, as progressive knowledge on FRK's role in the propagation of intracellular signalling cascades becomes available, further supporting investigations are anticipated to be conducted *in vivo*, employing specific repertoires of mouse-models with targeted gene disruptions or transgene introductions, akin to the types described above.

1.1.2.3 Src-related kinase lacking C-terminal regulatory tyrosine and N-terminal myristoylation sites (SRMS)

1.1.2.3.1 Gene discovery and organization

The third member of the FRK/PTK6 family, PTK70 or SRMS (Src Related tyrosine kinases lacking C-terminal Regulatory tyrosine and N terminal Myristoylation Sites) (pronounced "shrims") is the least characterized of the three and a relatively new protein kinase to be discovered. SRMS was first reported by Kohmura *et al.* in their attempt to elucidate the genes that regulate the growth and differentiation of neuroepithelial cells (Kohmura *et al.*, 1994). The gene encoding SRMS maps to chromosomal locus 20q13.33 in humans and 2.1 cM distal to locus D2Mit25 in mice which are conserved regions that lie adjacent to the PTK6 genetic locus (Kohmura *et al.*, 1994). That this gene maps to a region conserved

around PTK6, indicates that both genes may interact genetically. While SRMS bears functional units similar to the members of this family, its enzymatic activity, substrate specificity as well as its physiological function have not been characterized yet. The 54 kDa protein spanning 488 amino acids harbors an SH3, SH2 as well as a protein kinase domain; a structural framework common to both, BRK and FRK (Fig 1.10). However, unlike BRK and FRK, SRMS lacks a C-terminal regulatory region and has an extended N-terminal region (Fig 1.6). Furthermore, sequence homolgy also predicts K258 and Y380 as the potential ATP-contacting and autophosphorylation sites, respectively (Fig 1.10).

1.1.2.3.2 Expression in tissues

Through Northern blot analyses, SRMS expression was detected in the various murine tissues, with highest expression being observed in the lung, testes and liver (Kohmura *et al.*, 1994). While a moderate expression pattern was observed in murine spleen, kidney and ovary, the lowest level was seen in the intestine, muscle, thymus, heart, cerebellum and cerebrum (Kohmura *et al.*, 1994). A subsequent study also characterized the expression of SRMS in the murine skin with the highest detectable levels observed in the epidermis and in the murine keratinocyte cell lines as compared to those derived from melanomas (Kawachi *et al.*, 1997).

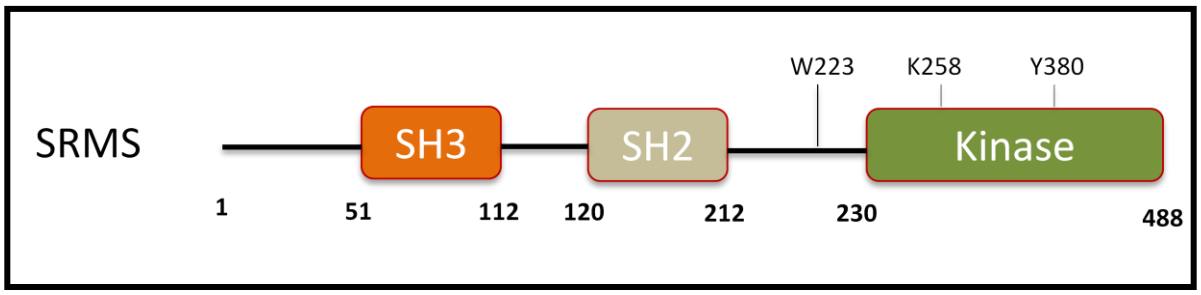

FIGURE 1.10: **Schematic representation of SRMS**. Schematic diagram of SRMS depicting its various functional domains; the SH3, SH2 and the kinase domains as well as the critical residues that are conserved with Src and FRK/PTK6 family kinases, and predicted to be involved in the regulation of tyrosine kinase activity. These are K262 and Y387 as the potential ATP-contacting and autophosphorylation sites, respectively. Also shown is W223, situated within the SH2-Kinase linker segment, which is highly conserved amongst Src family kinases as well as BRK and FRK and has been reported to be involved in the regulation of the active/inactive conformation of certain tyrosine kinases.

1.1.2.3.3 Substrates and binding partners

Intriguingly, while a multitude of substrates have been identified and extensively studied for BRK with a reasonable number of binding partners/substrates reported for FRK as well, no SRMS substrate has been characterized to date. However recently, Takeda *et al.*, described a proteomics study which revealed for the first time, the novel potential substrates of the Src family kinases including SRMS (Takeda *et al.*, 2010). The study was conducted with an aim to shed light on the substrate-specificity of the various human Src family kinases on a common platform. The research, predominantly based on a customized high-throughput *in vitro* kinase assay, examined a total of 519 unique substrates, utilizing mass spectrometry analysis for subsequent protein identification. The results of the comprehensive analysis categorized 11 prominent Src family Kinases including SRMS, into three groups; the first one indicating maximal substrate-specific interactions while the third one exhibiting limited substrate interactions. The potential substrate hits were ranked based on their relative substrate-specificity towards CRK (an adapter protein also designated as p38) as a common target. Although this study ascribed SRMS to the third group, a fair number of potential substrates were identified (Table 1.2). Dok1 was identified as one of the potential targets of SRMS in the highthroughput proteomic assay (Takeda *et al.*, 2010).

Recognition of the SRMS SH3/SH2 ligands may provide vital cues about the substrate selectivity pattern of SRMS. Information on the SH2 binding interactions of SRMS became available with a recent proteomics study (Zhao *et al.*, 2013) (Table 1.1). From a composite library of phosphotyrosine peptides screened against the SH2 domains of several tyrosine kinases, the SRMS SH2 domain recognition motifs were identified in ligands possessing hydrophobic residues at the P+3 position (where P is the phosphotyrosine). Specifically, it was noted that the SRMS SH2 domain favours binding to peptides distinctly possessing a phenylalanine or a methionine besides a cysteine residue common to BRK and FRK, at the P+3 position. At the P+1 position, the SRMS SH2 domain displays preferential selectivity for a histidine in addition to a glutamic acid residue as with BRK and FRK. Likewise, at the P-1 position, SRMS SH2 prefers a histidine or a tyrosine residue with the latter being a common choice, at the same position, for both BRK and FRK (Zhao *et al.*, 2013).

1.1.2.3.4 Mouse models

In an attempt to potentially uncover the physiological functions of the novel kinase, homozygous SRMS-knockout mice were generated. Such transgenic mice did not exhibit a change in the phenotype (Kohmura *et al.*, 1994). These SRMS-deficient mice were determined to be viable and fertile with normal off-springs and no apparent histological abnormalities in the body organs (Kohmura *et al.*, 1994). A similar observation was later made for BRK-deficient mice by Tyner and colleagues as well (Haegebarth *et al.*, 2006).

TABLE 1.2 List of the potential substrates of SRMS identified by Takeda *et al.*, 2010.

S.No	FLJ ID	Accession no.	Protein name	Category	Relative activity (CRK =1)	Rank
1.	FLJ95985	AK315002	**Tom1 like 1**		2.05	1
2.	FLJ31382	AK055944	**Dok1**	Adapter molecule	1.68	2
3.	FLJ54992	BC013200	**Tom1 like 1**		1.52	3
4.	FLJ80078	BC013200	**Lck**	Tyrosine Kinase	1.22	4
5.	FLJ42825	AK124815	**CRK associated substrate**	Adapter molecule	1.10	5
6.	FLJ81679	BC053532	**CRK associated substrate**	Adapter molecule	1.05	6
7.	FLJ81679	BC001718	**CRK**	Adapter molecule	1.00	7
8.	FLJ83777	BC065912	**ARG**	Tyrosine Kinase	0.84	8
9.	FLJ90539	AK075020	**SYK**	Tyrosine Kinase	0.43	9
10.	FLJ38194	AK095513	**Disabled 1**	Adapter molecule	0.39	10
11.	FLJ53493	AK295613	**HEF-like protein**	HisKa Domain	0.26	11
12.	FLJ23495	AK027148	**NEK11**	S/T kinase	0.26	12

1.2 Downstream of tyrosine kinase (Dok) family proteins

The Dok family of proteins comprises seven members; Dok1 –Dok7. That these proteins are phosphorylated downstream of several tyrosine kinases is a primary reason attributing to its nomenclature, Downstream of tyrosine kinases (Dok). The structures of all seven members of the Dok family remain highly conserved with each possessing two N-terminal functional domains, namely, a Pleckstrin homology (PH) domain and an Insulin receptor substrate (IRS) type phosphotyrosine binding (PTB) domain (Niu *et al.*, 2006; Shi *et al.*, 2004). The PH domain has a sequence length of about 120 amino acids and is organized into 2 anti-parallel Beta sheets together with a C-terminal amphipathic helix. Through its documented interactions with the phosphatidylinositol lipids of the biological membranes such as phosphatidylinositol (3,4,5)-trisphosphate and phosphatidylinositol (4,5)-bisphosphate (Wang and Shaw, 1995) as well as the beta-gamma heterotrimeric G proteins (Wang *et al.*, 1994) and protein kinase C (Yao *et al.*, 1994), the PH domain plays a role in recruiting proteins to different cellular membranes and enables their interaction with components of different signalling pathways. The IRS type PTB domain, on the other hand, is 112 amino acid residues long and is structurally similar to the PH domain in which a beta sandwich is capped by an alpha helix (Zhou *et al.*, 1996). PTB domains characteristically bind to phosphotyrosine-containing ligands with the sequence; NPXpY, where "X" represents any amino acid (Yaffe, 2002). IRS1 is known to bind to the autophosphorylated insulin receptor via the PTB domain which further leads to the phosphorylation of IRS1 and the subsequent recruitment of other SH2 containing signalling proteins (Yaffe, 2002).

Even as the structural topology of the seven Dok proteins remains extensively conserved, the genetic loci of the Dok family members are spatially unclustered across the human genome, suggesting limited genetic linkage between the respective Dok genes. The genetic loci and the corresponding protein size for each of the seven members are; Dok1: 2p13.1 (481 amino acids and 52.3 kDa), Dok2: 8p21.3 (412 amino acids and 45.3 kDa), Dok3: 5q35.3 (496 amino acids and 53.2 kDa), Dok4: 16q21 (326 amino acids and 37 kDa), Dok5: 20q13.2 (306 amino acids and 35.4 kDa), Dok6: 18q22.2 (331 amino acids and 38.3 kDa) and Dok7: 4p16.3 (504 amino acids and 53 kDa).

1.2.1 Cellular and physiological roles of Dok1 proteins

The Dok family proteins generally regulate a diverse set of physiological functions with Dok1-Dok3 functioning synergistically towards suppressing several intracellular signalling pathways thereby promoting a tumor suppressive role *in vivo*. (Berger *et al.*, 2010; Mashima *et al.*, 2009; Niki *et al.*, 2004; Yasuda *et al.*, 2004). The noted exception comes from a recent study that reported on the contrasting roles of Dok1 and Dok2 in CD200 receptor signalling (Mihrshahi and Brown, 2010). Upon CD200 ligand stimulation, Dok1 and Dok2 recruit diverse downstream proteins, with Dok1- CrKL (CT10 sarcoma oncogene cellular homologue-like) interaction negatively regulating the Dok2-mediated CD200R signalling cascade (Mihrshahi and Brown, 2010).

Dok4 was also identified as a negative regulator of ERK signalling as well as T-cell activation with its PH domain implicated in the cytoplasmic shuttling of the protein (Gerard *et al.*, 2009). Furthermore, the presence of a shortened C-terminal tail extending from the PTB domain as well as a rare single nucleotide polymorphism, R186H, within the same domain in Dok4, was shown to diminish its molecular interactions with Ret kinase and potentially yield a rare Dok4-related phenotype in humans, respectively (Hooker *et al.*, 2012). Dok5 expression, as determined by its transcriptional regulator FOXO3a, has been shown to correlate with cardiomyocyte differentiation (Wen *et al.*, 2009) and also promote neurite outgrowth in the mouse Neuro2A cells (Cai *et al.*, 2003; Grimm *et al.*, 2001). Additionally, Dok5, as a substrate of TrkB and TrkC (Tropomysin related kinase) also leads to the upregulation of the MAPK pathway upon neurotrophin stimulation (Shi *et al.*, 2006). Like Dok5, Dok6 is also a substrate of the TrkC receptor and has also been shown to promote Ret-mediated neurite outgrowth in murine cortical neurons (Li *et al.*, 2010).

The newest member of the Dok family, Dok7 was identified as a critical component in the muscle-specific tyrosine kinase (MuSK) mediated post-synaptic specialization of the neuro-muscular junction. *In vivo* studies demonstrated that mice deficient for Dok7 display impaired MuSK activation in muscle cells, thereby attesting to the role of Dok7 in the regulation of neuromuscular synapse formation (Okada *et al.*, 2006). Furthermore, a spectra of Dok7 mutations have also been identified in patients diagnosed with congenital myasthenia syndrome (CMS). Such mutations in the Dok7 gene have been reported to cause aggressive general impairments in the motor functions of the muscles including those of the eyes, jaws and limbs (Palace *et al.*, 2007; Srour *et al.*, 2010). Nonetheless, clinical treatment through the oral administration of Salbutamol, a β(2)-

adrenergic receptor agonist, has been used as a therapy against congenital myopathy with proven efficacy in adult and children (Burke *et al.*, 2013).

1.2.2 Downstream of tyrosine kinase 1

The Docking protein 1 (Dok1/p62) is a scaffolding protein which functions to mediate protein-protein interactions. Analogous to the other members of the family, the adapter protein is composed of an N-terminal PH domain and one IRS1 Type PTB domain besides a C-terminal Proline Rich region and several phosphorylation sites (Carpino *et al.*, 1997; Yamanashi and Baltimore, 1997) (Fig. 1.11). The gene encoding the approximately 53 kDa protein maps to the chromosomal locus 2p13.1, a region prone to rearrangement in several malignancies including leukemia (Saulnier *et al.*, 2012).

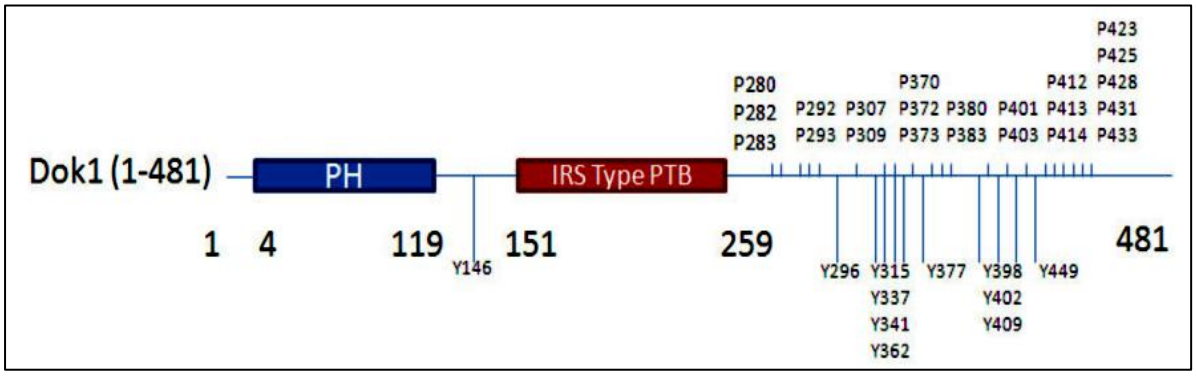

FIGURE 1.11: Schematic representation of Dok1: A schematic figure of the 481 amino acid Dok1 protein depicting the two functional domains, namely, the N-terminal situated Pleckstrin Homology (PH) Domain and the Insulin Receptor Substrate (IRS) type Phosphotyrosine binding (PTB) domain. The C-terminal segment of Dok1 is replete with proline and tyrosine residues.

Published data suggests Dok1 to be a phosphorylated protein downstream of several tyrosine kinases (Bhat *et al.*, 1998; Neet and Hunter, 1995; Wang *et al.*, 1995; Yamanashi and Baltimore, 1997). Dok1 was originally identified as a substrate of v-Src, c-Abl and p210-Bcr-Abl (Bhat *et al.*, 1998; Neet and Hunter, 1995; Woodring *et al.*, 2004a). The protein has been documented for its role as a key down-regulator of several tyrosine kinase signalling

pathways. For instance, Dok 1 is a negative regulator of cell-proliferation and lymphocyte signalling (Yamanashi *et al.*, 2000; Zhao *et al.*, 2001). The protein also inhibits mitogen-activated protein (MAP) kinase activity and mediates activin-induced apoptosis (Di Cristofano et al., 2001; Noguchi et al., 1999b; Yamakawa et al., 2002; Zhao et al., 2001). Additionally, Dok1 is known to promote cell growth and migration (Hosooka *et al.*, 2001). In mice, Dok1 was shown to suppress cell transformation and leukemogenesis (Di Cristofano *et al.*, 2001; Lee *et al.*, 2004a; Niki *et al.*, 2004). The cellular roles of Dok1 have been reported to be dictated by its cytoplasmic/membrane localization which is mediated by a characteristic nuclear export signal (NES) between amino acids 348-359 (Niu *et al.*, 2006).

Moreover, while prior reports suggest a cell transformation-suppressive role for Dok1 (Niki *et al.*, 2004), it was more recently demonstrated that oncogenic tyrosine kinases, p210 (Bcr-Abl) and v-Src, phosphorylate and target Dok1 for ubiquitin-mediated proteosomal digestion to stimulate cell transformation (Janas and Van Aelst, 2011). Hypermethylation of Dok1 has been reported to be a critical event mediating primary tumorigenesis in several cancer types (Saulnier *et al.*, 2012). Along these lines, a recent study suggested that Dok1 downregulation in epithelial ovarian cancer is attributed to promoter hypermethylation and that ectopic expression of the tumor suppressor results in better sensitivity to cisplatin treatment (Mercier *et al.*, 2011). Furthermore, the expression of the adapter protein was shown to be regulated mainly by a transcription factor, E2F1 for which there exists three response elements, designated E2F1 response elements (ERE) within the core promoter region of Dok1. It was also noted that E2F1 recruitment to the promoter region was hampered by DNA methylation (Siouda *et al.*, 2012).

Other etiological implications of Dok1 have also been reported. Recent reports demonstrated that Dok1 deficiency augments a physiological reduction in bone mineral density, a condition referred to as Osteopenia (Kawamata *et al.*, 2011). Murine models of asthma have demonstrated a therapeutic potential of Dok1 in treating allergic respiratory diseases. Specifically, Dok1 was shown to negatively regulate allergen-induced Th2 inflammation, mucus production and airway hyper-responsiveness in mice models of asthma (Lee *et al.*, 2012). Dok1 expression is also known to exert direct effects on the immune system. For instance, ectopic expression of Dok1 interferes with the development of T-cells, thus causing the accumulation of an innate-like sub-population of eomesodermin expressing CD8+ T cells (Besin *et al.*, 2012). A computational platform based on such parameters as cellular protein concentration, supported a model for Dok1-integrin complex formation under

low cellular concentration of the cytoskeletal protein, talin (Geier *et al.*, 2011). The model, thus, endorses a potential role of Dok1 in integrin receptor-mediated signalling (Geier *et al.*, 2011).

With such physiologically diverse functions, Dok1 presents itself as an exciting target to pursue for examining further implications, if any, on the vast repertoire of the cell's intrinsic signalling pathways. More importantly, Dok1 has recently garnered widespread attention for its potential role as a tumor suppressor in a variety of cancers. In a recent proteomics study, Dok1 was identified as a potential substrate of SRMS (Takeda *et al.*, 2010). However, Dok1 has yet to be validated as a cellular target of SRMS and the functional link between Dok1 and SRMS has not been established. This thesis, in part, describes the characterization of Dok1 as a substrate of SRMS.

1.3 Breast cancer clinical subtypes

Breast cancer is divided into several clinical subtypes that include ductal carcinoma in-situ (DCIS), infiltrating ductal carcinoma (IDC), lobular carcinoma in-situ (LCIS), infiltrating lobular carcinoma (LC), as well as into different stages from 0 to IV (based on tumor size and metastatic propensity) (Fadare and Tavassoli, 2008; Guiu *et al.*, 2012). Primary breast tumors are classified into at least five different molecular types based on gene expression profiling and immunohistochemistry (Fadare and Tavassoli, 2008; Guiu *et al.*, 2012). They include i) normal-like breast cancers; ii) basal-like breast cancers, which are negative for estrogen receptor (ER), progesterone-receptor (PR), and human epidermal growth factor receptor 2 (HER2 or ERBB2) overexpression iii) luminal-A cancers, which are mostly ER-positive and histological low in grade; iv) luminal-B cancers, which are also mostly ER-positive but may express low levels of hormone receptors and are often high-grade; v) HER2-positive cancers, associated with aggressive tumor behavior; and vi) Claudin-low, Basal-like) (Fadare and Tavassoli, 2008; Guiu *et al.*, 2012; Prat *et al.*, 2013). Of note is that a recent integrated analysis of copy number and gene expression in a total of about 2000 primary breast tumours led to the reclassification of breast cancers into 10 subgroups (Curtis *et al.*, 2012).

2. Hypothesis:

Dok1 possesses several proline and tyrosine residues and was also identified as a potential substrate of SRMS in a proteomics study (Takeda *et al.*, 2010). We, therefore, hypothesize that Dok1 is a substrate of SRMS and associates with SRMS via SH3 and/or SH2 interactions. The expression profile in breast cancer as well as the biochemical characterization of BRK and FRK have been extensively studied (Barker *et al.*, 1997; Berclaz *et al.*, 2000; Cance *et al.*, 1994; Meyer *et al.*, 2003; Qiu and Miller, 2002). Since SRMS is a novel tyrosine kinase that remains largely understudied, it was imperative that this study included the characterization of the tyrosine kinase. Therefore, the following specific aims were developed towards testing the broad hypothesis:

2.1 To determine the expression and sub-cellular localization of SRMS in various human breast cell lines.

SRMS is a non-receptor tyrosine kinase belonging to the BRK kinase family (Serfas and Tyner, 2003). There exists a significant divergence in a multitude of cellular characterictics among breast cancer cell lines (Neve *et al.*, 2006). While the expression of BRK and FRK has been determined in several breast-derived cell lines, that for SRMS is as yet unknown. The expression pattern of SRMS shall be determined via Western blotting in nine select human breast cell lines:- a non-tumorigenic breast epithelial cell line; 184B5 and eight breast cancer cell lines namely, BT20, MCF7, MDA-MB-231, MDA-MB-435, MDA-MB-468, HBL100, SKBR3 and AU565. These breast cancer cell lines express different hormone receptors (estrogen, progesterone, HER2 or triple-negative) on their cell membranes, and shall thus contribute to the heterogeneity of the study based on hormone receptor status (Table 2.1). Sub-cellular localization of SRMS in selected cell lines shall also be investigated using immunocytochemistry techniques.

TABLE 2.1: Classification of breast cancer cell lines according to their clinical and molecular characteristics including hormone receptor status and site of primary tumor and invasive capacity. The breast cancer cell lines used in the study express either the estrogen (ER), progesterone (PR) or HER2 receptors (HER2) or none of these three (triple negative breast cancers or TNBC). Based on the site of primary tumor and cell type, these breast cancer cell lines are also classified as adenocarcinoma (AC) or invasive ductal carcinoma (IDC). HBL-100 is a cell line derived from normal tissues (N) isolated from the breast milk (BM).

S. No	Cell Line	ER	PR	HER2	TNBC	Derived tumor type
1	BT-20				+	IDC
2	MCF7	+	+			IDC
3	MDA-MB-231			+		AC
4	MDA-MB-435				+	IDC
5	MDA-MB-468				+	AC
6	HBL-100	+				N,BM
7	SKBR3			+		AC
8	AU565			+		AC

2.2 To validate the activity of SRMS and generate a series of SRMS mutants to study catalytic regulation and effect on sub-cellular localization.

Owing to extensive sequence homology, SRMS retains a notably conserved framework of functional domains critical for mediating protein-protein interactions (SH3/SH2 domains) as well as for sustaining a catalytic potential (Kinase domain) as observed in the other members of its family, BRK and FRK. Furthermore, certain amino-acids remain conserved as observed through the CLUSTAL W multiple sequence alignment tool. Using site-directed mutagenesis and conventional cloning strategies, several point mutations and deletions shall be introduced within the SRMS sequence to study catalytic regulation. Additionally, the

repercussions of these mutations on influencing the sub-cellular localization of SRMS shall also be studied via fluorescence microscopy. Specifically, these mutations include:

I. Y380F - Predicted autophosphorylation site.
II. K258M - Residue predicted to be involved in ATP-binding.
III. W223A - Residue predicted to be involved in intra-molecular associations with the kinase domain.
IV. R147A - SH2 domain residue predicted to be involved in phosphotyrosine binding.
V. P214A - SH2-kinase linker residue predicted to be involved in intra-molecular associations.
VI. P218A+P226A (2PA SRMS) - SH2-kinase linker residue predicted to be involved in intra-molecular associations.
VII. N-terminus deleted SRMS (ΔN-SRMS)
VIII. Partial N-terminus deleted SRMS (PΔN-SRMS)
IX. Deletion SH3 SRMS (ΔSH3-SRMS)
X. Deletion SH2 SRMS (ΔSH2-SRMS)

2.3 To determine the expression and localization of Dok1 in selected cell lines.

To date, there exists no information on the expression of Dok1 in breast tissue-derived cell lines. However, since Dok1 is a putative tumor suppressor known to be down-regulated in several cancer types, it is critical that the expression profile of the same be determined in breast cancer. The expression profile of Dok1 shall be determined in selected cell lines (as mentioned above) via Western blotting and sub-cellular-localization studied via immunocytochemistry and sub-cellular fractionation.

2.4 To determine the molecular interactions between SRMS and Dok1 through GST-pulldown assays.

A recent proteomics study determined Dok1 to be a potential target of the tyrosine kinase, SRMS (Takeda *et al.*, 2010). However, Dok1 is yet to be validated and characterized as a SRMS substrate. GST-pull down assays shall be utilized towards ascertaining SH3/SH2 dependent interactions of SRMS with Dok1. Recombinant GST-fused SRMS SH3 and SH2

proteins shall be used to investigate the molecular association of SRMS with Dok1. Furthermore, since Dok1 has several potential SH3-binding ligands along its extended C-terminus segment, C-terminal truncated mutants of Dok1 shall be generated and binding with SRMS studied via co-immunoprecipitation studies.

2.5 To validate Dok1 as a substrate of SRMS.

Validation of Dok1 as a substrate of SRMS shall be carried out via immunoprecipitation and *in vitro* kinase assays. Furthermore, co-localization analyses of SRMS and Dok1 *in vivo*, via immunocytochemistry shall be employed as well.

3. Materials and methods:

3.1 Reagents and chemicals

The reagents and chemicals used towards the experiments described in this thesis have been compiled in Table 3.1.

TABLE 3.1 List of Reagents and Suppliers

Reagent/ Chemicals	Supplier and Catalogue numbers.
2x Laemmli	Sigma-Aldrich, S3401
Acrylamide	Sigma-Aldrich, A5934
Acetic Acid	Fisher Scientific, A465250
Agar	Fisher Scientific, BP1425-2
Agarose	Fisher Scientific, BP1360
Albumin (Bovine)	Amresco, 0332
Aprotinin	Sigma-Aldrich, A6279
Ampicillin	EMD Millipore, 171255
Ammonium Persulfate (APS)	Sigma-Aldrich, A3678
Adenosine triphosphate (ATP) 10mM	New England Biolabs, P0756S
Bovine Calf Serum	Thermo Scientific, SH30087.02
Bisacrylamide N'N-methylene	Amresco, 0172
Boric acid	EMD Millipore, 203-667
Coomassie Brilliant blue	G Biosciences, 786-497
Disodium Phosphate (Na_2HPO_4)	Fisher Scientific, S375-500
Dimethyl Sulfoxide (DMSO)	Fisher Scientific, TS-20684
Dulbecco's modified Eagle's medium (DMEM)	Thermo Scientific,SH30023.01
Ethylenediaminetetraaceticacid (EDTA	EMD Millipore, 324503
Glutathione Sepharose beads	Novagen, 70541
Gel Red	Biotium, 41002
Glycerol	Sigma-Aldrich, G5516
Glycine	Fisher Scientific, S80028
Ethyl alcohol (EtOH)	Fisher Scientific, S25310
ECL (Enhanced Chemiluminescence solution)	Perkin Elmer, NEL103001EA
Hydrochloric Acid (HCl)	Fisher Scientific, A508-P500

HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)	Sigma-Aldrich, H3375
Kanamycin	EMD Millipore, 420311
Methanol	EMD Millipore, MX0475
Magnesium Chloride ($MgCl_2$)	Fisher Scientific, 232-094-6
Manganese Chloride ($MnCl_2$)	Fisher Scientific, M87-500
Monopotassium Phosphate (KH_2PO_4)	Fisher Scientific, BP-362-500
Nitrocellulose Membrane	Pall Life sciences, 66489
Penicillin /Streptomycin	Fisher Scientific, BP2959-50
Potassium Chloride (KCl)	Fisher Scientific, AC193780000
Polyethyleneimine (PEI)	Sigma-Aldrich, 408727
Protein A beads	Santacruz Biotech, sc-2001
Protein G beads	Santacruz Biotech, sc-2002
Phenylmethylsulfonylfluoride (PMSF)	Fisher Scientific, 329-98-6
Sodium Dodecyl sulfate (SDS)	Sigma-Aldrich, L3771
Sodium Azhide (NaN_3)	Fisher Scientific, S227-100
Sodium Orthovanadate ($NaVO_4$)	EMD Millipore, 567540
Skim Milk	Carnation
TEMED ((N,N,N´,N´-tetramethylethylenediamine)	Sigma-Aldrich, T9281
Tris Base	Fisher Scientific, BP152-5
Triton X-100	Fisher Scientific, AC215680000
Tryptone	EMD Millipore, BP1421-500
Tween-20	Fisher Scientific, BP337500
Yeast Extract	Fisher Scientific, BP9727-2

TABLE 3.2 Names and Addresses of Suppliers

Supplier	Address
Fisher Scientific	Walton, Massacheusetts, USA
Sigma-Aldrich	St. Louis, Missouri, USA
EMD Millipore	Cambridge, United Kingdom
EMD Chemicals	San Diego, California, USA
Invitrogen Life Technologies	Green Island, New York, USA
Santacruz Biotechnologies	Santa Monica, California, USA

Thermo Scientific	Logan, Utah, USA
Biotium	Burlington, Ontario, Canada
Bio-Rad Inc.	Hercules, California, USA
Amresco Inc.	Solon, Ohio, USA
New England Biolabs	Ispwich, Massacheusetts, USA
Perkin Elmer	Waltham, Massacheusetts, USA

3.2 Expression vectors

All PCR amplifications were performed using *Econotaq* polymerase supplied with *Taq* buffer and dNTP mix (300031-1, Lucigen) with the following reaction and cycling conditions:

Reactions Components (50 µl reaction):

10X Standard *Taq* Reaction Buffer with Mg^{2+} (1X), 10 mM dNTPs (200 µM), 10 µM Forward Primer (0.2 µM), 10 µM Reverse Primer (0.2 µM), Template DNA (<100 ng), *Taq* DNA Polymerase (1.25 units), Nuclease-free water (to 50 µl).

Cycling Conditions:

1. Initial Denaturation	95°C (30 seconds)
2. Extension (30 Cycles)	95°C (30 seconds)
	45-68°C (30-60 seconds)
	68°C (1 min/kb)
3. Final Extension	68°C (5 min.)

3.2.1 SRMS expression vectors

All primers used towards PCR reactions were purchased from Invitrogen Life Technologies, (New York, USA). The full length SRMS cDNA amplified from the pANT7-cGST-SRMS plasmid, purchased from DNASU Plasmid Repository (Tempe, AZ) via PCR was cloned C-terminal to the GFP sequence into the *Hind*III and *Bam*HI sites of the pEGFP-C1 plasmid using the oligonucleotide primers shown in Table 3.3. The same primers were also used to clone the SRMS cDNA C-terminal to the mCherry sequence, into the same sites of the pmCherry-C1 plasmid (a kind gift from Dr Scot Stone, Department of Biochemistry, University of Saskatchewan). All other deletion variants of SRMS were generated using the

GFP-SRMS construct as the template employing single or double pairs of primers as shown in Table 3.3.

GST-SRMS constructs were generated by cloning the SRMS-SH3 and SH2 cDNAs into the BamHI and XhoI sites located within the multiple cloning site, C-terminal to the GST sequence, in the pGEX-6-p-3 vector backbone [Table 3.3]. All constructs were verified via sequencing (sequencing facility: NRC-PBI, University of Saskatchewan, Saskatoon.)

3.2.1.1 Site directed mutagenesis was employed to introduce point mutations in the SRMS sequence via the commercially available QuikChange site-directed mutagenesis (Stratagene) kit, according to the manufacturer's protocol as outlined below:

Reaction Components:

Standard Reaction Buffer with Mg^{2+} (1X), 20 ng Template DNA, 125 ng forward primer , 125 ng reverse primer, 200 μM dNTP mix, nuclease free water (to 50 μl) and *Pfu turbo* DNA polymerase (1.25 units).

Cycling Conditions:

1. Initial Denaturation	95°C (30 seconds)
2. Extension (18 Cycles)	95°C (30 seconds)
	55°C (30-60 seconds)
	68°C (1min/kb)
3. Final Extension	68°C (5 min.)

The PCR-mutagenesis products were incubated with the restriction enzyme, *Dpn*I at 37 °C for 1 hour, to digest the parental cDNA in the PCR product and the digested samples directly used for transforming the commercially procured X-Gold ultra-competent cells (#200315, Agilent Technologies). The following day, independent bacterial colonies were picked, DNA extracted from the clones and screened via sequencing (sequencing facility: NRC-PBI, University of Saskatchewan, Saskatoon.)

TABLE 3.3: List of SRMS expression constructs generated via cloning and site-directed mutagenesis.

S. No	DNA Construct	Restriction Sites	Primers
1.	GFP-SRMS	*Hind*III, *Bam*HI	5′-CTC AAG CTT CGG AGC CGT TCC TCA GGA GGC G-3′ 5′-CCG GGA TCC TCA GGG GTG GCA TCT GGT GGA-3′
2.	mCherry-SRMS	*Hind*III, *Bam*HI	5′-CTC AAG CTT CGG AGC CGT TCC TCA GGA GGC G-3′ 5′-CCG GGA TCC TCA GGG GTG GCA TCT GGT GGA-3′
3.	GFP-ΔN-SRMS	*Hind*III, *Bam*HI	5′-CTC AAG CTT CGG AGC CGT TCC TCA GGA GGCG-3' 5′-CCG GGA TCC TCA GGG GTG GCA TCT GGT GGAT-3′
4.	GFP-PΔN-SRMS	*Hind*III, *Xho*I *Bam*HI	5′-CTC AAG CTT CGC CCT TCC CTC AGC TCT TCC TT-3′ 5′-AAA CTC GAG GCC ATG GTC CGG CTC GCC-3′ 5′-AAA CTC GAG CCC CAG AAG GCC CCG AGG-3′ 5′-CCG GGA TCC TCA GGG GTG GCA TCT GGT GGA-3′
5.	GFP-ΔSH2-SRMS	*Hind*III, *Xho*I *Bam*HI	5′-CTC AAG CTT CGG AGC CGT TCC TCA GGA GGCG-3′ 5′-AAA CTC GAG TTG GTC TGA GAG CGT CTC-3′ 5′-AAA CTC GAG CCC CAG AAG GCC CCG AGG-3′ 5′-CCG GGA TCC TCA GGG GTG GCA TCT GGT GGAT-3′
6.	GFP-ΔSH3-SRMS	*Hind*III, *Xho*I *Bam*HI	5′-CTC AAG CTT CGG AGC CGT TCC TCA GGA GGCG-3′ 5′-AAA CTC GAG GCA AGG CTC GGC GGG GAG-3′ 5′-AAA CTC GAG ACG CTC TCA GAC CAA CCC-3′ 5′-CCG GGA TCC TCA GGG GTG GCA TCT GGT GGAT-3′
7.	GST-SRMS-SH2	*Bam*HI, *Xho*I	5′-CTG GGA TCC GAG ACG CTC TCA GAC CAA CCC-3′ 5′-CGG CTC GAG TCA CCT CGG GGC CTT CTG GGG-3′
8.	GST-SRMS-SH3	*Bam*HI, *Xho*I	5′-CTG GGA TCC CCC GCC GAG CCT TGC AGC CCC-3′ 5′-CGG CTC GAG TCA GTC TGA GAG CGT CTC AGG-3′
9.	GFP-SRMS-K258M	*Hind*III, *Bam*HI	5′-CCC GTG GCG ATC ATG GTC ATC AAG TCA-3′ 5′-TGA CTT GAT GAC CAT GAT CGC CAC GGG -3′
10.	GFP-SRMS-W223A	*Hind*III, *Bam*HI	5′-AGG CAG GAC GTG GCT GAG CGG CCA CAC-3′ 5′-GTG TGG CCG CTC AGC CAC GTC CGT CCT-3′
11.	GFP-SRMS-Y380F	*Hind*III, *Bam*HI	5′-AAG GAC GAC ATC TTT TCC CCG AGC AGC-3′ 5′-TTC CTG CTG TAG AAA AGG GGC TCG TCG-3′

3.2.2 Dok1 expression vectors

GFP-Dok1 construct was a kind gift from Dr. Bakary S. Scylla, Lyon, France. Five Dok1 deletion mutants were generated using the GFP-Dok1 construct as the template. Using the same PCR conditions as mentioned above, five pairs of primers were used to amplify five Dok1 cDNA variants differing progressively in length and cloned C-terminal to the GFP sequence in the EcoRI and SmaI sites of the pEGFP-C1 vector backbone [Table 3.4]

The GFP-Dok1 template was also utilized, via PCR, to clone Dok1 C-terminal to the GST cDNA sequence into the EcoRI and NotI sites of the pGEX-5-x-3 vector, using the oligonucleotide primers shown in Table 3.4. All constructs were verified via sequencing (sequencing facility: NRC-PBI, University of Saskatchewan, Saskatoon.)

TABLE 3.4: List of Dok1 expression constructs generated.

S. No	DNA Construct	Restriction Sites	Primers
1.	GFP- DokΔ1	*EcoR*I, *Sma*I	5′-AGT GAA TTC GGA CGG AGC AGT GAT GGA A-3′ 5′-ATT CCC GGG TCA AGT CTC AAC TGC CTG-3′
2.	GFP- DokΔ2	*EcoR*I, *Sma*I	5′-AGT GAA TTC GGA CGG AGC AGT GAT GGA A-3' 5′-ATT CCC GGG TCA CTT CCG TTG TAC TCC-3′
3.	GFP- DokΔ3	*EcoR*I, *Sma*I	5′-AGT GAA TTC GGA CGG AGC AGT GAT GGA A -3′ 5′-ATT CCC GGG TCA CTT GGC CTT CAG CAA-3′
4.	GFP- DokΔ4:	*EcoR*I, *Sma*I	5′-AGT GAA TTC GGA CGG AGC AGT GAT GGA A-3′ 5′-ATT CCC GGG TCA CTT CAC CCG AGC TTG-3′
5.	GFP- DokΔ5	*EcoR*I, *Sma*I	5′-AGT GAA TTC GGA CGG AGC AGT GAT GGA A-3′ 5′-ATT CCC GGG TCA CTT GGG AGC AAG GAG-3′
6.	GST-Dok-C-Term	*EcoR*I, *Not*I	5′- ATA GAA TTC CGA CGG AGC AGT GAT GGA A -3′ 5′- ATA GCG GCC GCT CAG GTA GAG CC -3′

3.3 Cell lines and cell culture:

HEK293, HeLa, BT20, MCF7, MDA-MB-231, MDA-MB-435, MDA-MB-468, AU565, SKBR3 and HBL100 were purchased from the American Tissue Type Culture Collection (ATCC, Manassas, VA, USA). All cell lines were maintained in High glucose (4.5g/L) Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% bovine calf serum (Thermo Scientific, Logan, USA), 4 mM L-Glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin (Sigma Aldrich, St. Louis, MO, USA). The required personal protective equipment was used for the purpose. Waste solution comprising exhausted media, trypsin or other liquids, was de-contaminated with 30% bleach solution prior to disposal. All other forms of waste including gloves, plastic pipettes, Pasteur pipettes etc. were decontaminated with bleach solution, sealed in autoclavable double bags and sent for disposal. The working area was decontaminated with 70% ethanol before and after use.

Generally, the HEK293 cell line was chosen for the purpose of characterizing the biochemical properties of SRMS including its sub-cellular localization and substrate-specificity towards Dok1 via immunoprecipitation analyses. HEK293 is an immortalized cell line isolated from the human embryogenic kidneys that expresses very low level of SRMS. The relative ease associated with the growth and maintenance of the cell line as well as high transfection efficiency and high protein production were inherent traits that were deemed useful towards the use of the cell line for the aforementioned experiments.

3.3.1 PEI-mediated transfection:

All transfections were carried out in the HEK293 cell line, cultured as described above. Cells, cultured in 6 well plates, were transiently transfected with a total of 2.5 µg DNA using 1% Polyethyleneimine 'Max' (PEI) (Polysciences Inc., Warrington, PA, USA) at a DNA to transfection reagent ratio: 1:3. For each well in the 6-well plate, 2.5 µg of the appropriate DNA was mixed with 107.5 µl of 0.15 M sterile NaCl via gentle vortexing for 10 seconds. 15 µl of the transfection reagent, PEI, was then added to this mixture followed by another 10 seconds of gentle vortexing. DNA-PEI complex formation was allowed to take place by incubating the mixture at room temperature for 10 minutes followed by dispensing it dropwise into the wells. The cells were incubated for 24 hours post transfection, transfection efficiency checked the following day using the Olympus 1x71 inverted fluorescence microscope and cells harvested for use towards the experiment.

3.4 SDS-PAGE and Western Blotting:

3.4.1 SDS-PAGE:

Whole cell lysates were prepared directly in 2x Laemmli (Sigma-Aldrich). Total cell lysates were prepared in lysis buffer comprising 20 mM Tris pH 7.5, 1% Triton X-100, 150 mm NaCl, protease inhibitors: Aprotinin 5 mg/l and 0.1 mM PMSF as well as 0.3 mM sodium orthovanadate. Protein samples derived from either lysates (whole cell lysates/ total cell lysates) or immunoprecipitates, were resolved via sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE). SDS-PAGE was carried out using the Mini-PROTEIN 4 gel electrophoresis system (Bio-Rad-165). 1.5 mm gels were cast using the appropriate glass plates designed and manufactured to include a permanently articulated spacer (Bio-Rad). 10% polyacrylamide gels were used for all experiments. The resolving gel comprises 29.2% acrylamide, 0.8% bis-acrylamide, 0.4% SDS, 375 mM Tris HCl pH 8.8, 0.16% ammonium persulfate (APS), 0.1% N,N,N',N' Tetramethylenediamine (TEMED) and H_2O. The stacking gel comprises 29.2% acrylamide, 0.8% bis-acrylamide, 0.4% SDS, 125 mM Tris HCl pH 6.8, 0.24% (w/v) ammonium persulfate (APS), 0.1% (w/v) N,N,N',N' Tetramethylenediamine (TEMED) and H_2O.

Pre-stained protein ladder (NEB, #P7711S) and the protein samples (boiled at 100 °C in 2x laemmli buffer for 5 minutes) were loaded onto the gels (10 wells or 15 wells) and the samples electrophoresed in 1x SDS running buffer at a constant voltage of 150 volts for 1 hour or for the duration required for the bromophenol blue dye front to completely pass through the gel.

3.4.2 Western Blotting:

Following electrophoresis, the gels were carefully removed from the glass plates, the stacking gel discarded and the resolving gel placed over 5 sheets of 3 MM filter papers (with dimensions equalling the length and breadth of the gel) pre-soaked in transfer buffer. Nitrocellulose membrane, soaked in transfer buffer and cut to the dimensions of the gel was placed above the gel and over which an additional 5 sheets of 3 MM filter papers were stacked to yield a composite "sandwich-like" assembly. The assembly was placed into the gel holder cassette and the cassette inserted into the Western blotting apparatus (Bio-Rad) filled with transfer buffer (refer to Table 3.5) up to the manufacturer's indicated mark.

Protein transfer was allowed to proceed for 1 hour at a constant voltage of 100 volts at 4 degrees.

Following the completion of the transfer, the nitrocellulose membranes were removed and subject to blocking via incubation with blocking buffer (refer to Table 3.5) at room temperature for 40 minutes. The blot was then washed briefly with 1x TBST buffer (refer to Table 3.5) and incubated with the appropriate primary antibody (as enlisted below) diluted at a ratio of 1:1000 in a primary antibody buffer (refer to Table 3.5) and incubated overnight at 4°C on a rocker. The following day, the membrane was washed for 15 minutes three times with 1x TBST buffer. The membrane was then incubated with the appropriate secondary antibody (HRP-conjugated goat anti mouse/ goat anti rabbit, as mentioned below) at a dilution of 1:10000 in a secondary antibody buffer (refer to Table 3.5) at 4°C on a rocker for 40 minutes. The membrane was subsequently washed three times with 1x TBST, incubated with Enhanced chemiluminiscence solution (ECL) (Perkin Elmer, Netherlands) for 1 minute and the immunoreactive proteins detected via exposing the membranes to X-ray films.

3.4.2.1 Primary and Secondary Antibodies

All primary and secondary antibodies were purchased from Santa-Cruz Biotechnology Inc., Santa Cruz, CA, USA. Primary antibodies include: anti-SRMS (sc-68341), anti-GST (sc-33613), anti-β-tubulin (sc-9104), anti-GFP (sc-8334) and anti-phosphotyrosine (sc-508). Anti-Dok1 was a kind gift from Dr. Ryuji Kobayashi (University of Texas, Austin, USA). The anti-Sam68 (AD1) polyclonal antibody has been previously described (Chen *et al.*, 1999). The working concentrations/dilutions of the primary antibodies are indicated in Table 3.5. Secondary antibodies used towards Western blotting include: goat HRP-conjugated secondary antibodies against rabbit (sc-2004) and mouse (sc-2005). Secondary antibodies, used towards immunocytochemistry experiments include: goat anti-rabbit IgG-FITC (sc-2012) and goat anti-mouse IgG-Texas Red (sc-2979). Working dilutions of the secondary antibodies are indicated in Table 3.6.

TABLE 3.5 List of media/buffers and their composition

BUFFER/ MEDIA	COMPOSITION
1X PBS	137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.46 mM KH_2PO_4, pH 7.4 .
1x TBST	150 mM NaCl, 10 mM Tris pH 8.0, 10% Tween-20 (final at 0.1% v/v)
Blocking buffer	5% Skim milk in TBST buffer (5 g Skim milk in 100 mL TBST buffer)
Primary antibody buffer	0.1% Tween-20, 5% BSA in 1x TBS
Secondary antibody buffer	0.1% Tween-20, 5% BSA in 1x TBS
1X Tris buffered saline (TBS)	150 mM NaCl, 10 mM Tris pH 8.0
Western blotting Transfer buffer	25 mM Tris, 192 mM glycine, pH 8.3, 20% methanol.
SDS PAGE Running buffer	25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3.
Mammalian cell lysis buffer	20 mM Tris pH 7.5, 1% TritonX-100, 150 mM NaCl, protease inhibitors: Aprotinin 5 mg/L, 0.1 mM PMSF, phosphatase inhibitor: 0.3 mM sodium orthovanadate.
Bacterial cell lysis buffer	50 mM Tris 7.5, 150 mM NaCl, 1% TritonX-100
2XYT Media for bacterial cultures	1.0% Tryptone, 0.5% Yeast Extract, 1.0% NaCl, pH 7.0

TABLE 3.6 List of chemicals/reagents and their composition

CHEMICALS/REAGENTS STOCK	COMPOSITION
10 mg/mL Kanamycin	0.5 g 50 mL ddH_2O (double distilled H_2O).
50 mg/mL Ampicillin	0.5 g in 10 mL ddH_2O.
1 M IPTG (Isopropyl β-D-1-thiogalactopyranoside)	1 g IPTG dissolved in 4.2 mL ddH_2O, filtered through 0.22 μm.
100 mM PMSF (Phenylmethylsulfonylfluoride)	1.74 g PMSF in 100 mL Isopropanol.

TABLE 3.7: Working concentrations/dilutions of primary antibodies used in Western blotting (WB) and immunocytochemistry (IC) experiments.

Primary antibody	Working Concentration/Dilution
Anti-SRMS (sc-68341)	0.2 μg/mL (WB), 2 μg/mL (IC)
Anti-GST (sc-33613)	0.2 μg/mL (WB)
Anti-β-tubulin (sc-9104)	0.2 μg/mL (WB)
Anti-GFP (sc-8334)	0.2 μg/mL (WB)
Anti-phosphotyrosine (sc-508)	0.2 μg/mL (WB)
Anti-Dok1	1:2500 (WB), 1:300 (IC)
Anti-Sam68 (rabbit) (AD1)	1:1000 (WB)

TABLE 3.8: Working dilution range of secondary antibodies used towards Western blotting and immunocytochemistry experiments.

Secondary antibody	**Working Dilution**
Goat anti-rabbit IgG-HRP (sc-2004)	1:10000 (WB)
Goat anti-mouse IgG-HRP (sc-2005)	1:10000 (WB)
Goat anti-rabbit IgG-FITC (sc-2012)	1:200 (IC)
goat anti-mouse IgG-TR (sc-2979)	1:200 (IC)

3.5 Immunocytochemistry:

Cells, seeded on coverslips, were cultured in 6-well plates, fixed with 1% paraformaldehyde in 1x PBS (Phosphate buffered saline), pH 7.4, for 5 minutes and permeabilized with 0.5% Triton X-100 in PBS for 5 min at room temperature, and incubated with anti-SRMS or anti-Dok1 antibodies (dilution factor- 1:200) for 1 h in PBS at room temperature. The cells were washed with 0.1% Triton X-100 in PBS and incubated with the appropriate secondary antibodies (dilution factor- 1:200) in PBS for 30 minutes. Goat anti-mouse coupled to Texas Red (Sc-2781, Santa Cruz Inc.) and goat anti-rabbit coupled to FITC (Sc-2012, Santa Cruz Inc.) were used as secondary antibodies. The coverslips were then mounted onto glass slides with glycerol containing 3 mg/mL 4',6-diamidino-2-phenylindole (DAPI) to stain the nuclei. For cells transfected with the GFP-fused constructs, following cell permeabilization, the coverslips were directly mounted onto glass slides with glycerol containing DAPI for nuclei staining. The cells were observed under an Olympus 1X-71 inverted microscope and the images captured at 60x magnification using the "Infinity Analyze" software platform (Lumina Inc.).

3.6 Immunoprecipitation

All transfections were carried out in the HEK293 cell line, cultured as described above. Cells washed in cold 1x phosphate-buffered saline (PBS), were lysed in freshly prepared lysis buffer constituting 20 mM Tris pH 7.5, 1% TritonX-100, 150 mm NaCl, protease inhibitors: Aprotinin 5 mg/L and 0.1 mM PMSF as well as 0.3 mM sodium orthovanadate (Enzo life sciences). Lysates were prepared by incubating the harvested cells in ice-cold lysis buffer for 30 minutes followed by centrifugation for 10 minutes at 13k xg. Supernatants were collected and transferred into fresh tubes and incubated with 1ug of the appropriate antibody and maintained on a gyrorotator for 1 hour at 4°C. 20 µl of Protein A agarose beads were then added to the samples and incubated for another 40 minutes on the gyrotator at 4°C. The beads were washed twice with ice-cold lysis buffer and 1x PBS before the immunoprecipitated proteins were denatured in 2x Laemmli buffer via boiling at 100°C for 5 minutes and resolved via SDS-PAGE.

3.7 Sub-cellular fractionation

Sub-cellular fractionation was carried out using the ProteoExtract Sub-cellular Proteosome Extraction Kit (#539790, Calbiochem, San Diego CA, USA) as instructed by the manufacturer. All buffers/solutions were provided in the kit. Briefly, cells grown in 100 mm culture plates were harvested using the manufacturer's "wash buffer" and lysed in an eppendorf tube using 1 mL of "extraction buffer I". Cellular lysis was allowed to proceed for 10 minutes at 4°C. The lysate was then subjected to centrifugation at 1000 xg for 10 minutes at 4°C. The supernatant obtained was collected as the cytosolic fraction. Subsequently, 1 mL of "extraction buffer II" was added to the remaining cellular components and the mixture left to incubate at 4°C for 30 minutes. These lysates were then subjected to centrifugation at 6000 xg for 10 minutes at 4°C. The supernatant was collected as the membrane fraction. Finally, 500 µl of "extraction buffer III" was added to the remaining cellular components and the mixture incubated for 10 minutes at 4°C. The lysate was subjected to centrifugation at 6800 xg for 10 minutes at 4°C. The supernatant obtained was collected as the nuclear fraction. Proteins from the three cellular fractions were eventually resolved via SDS-PAGE and detected using the appropriate antibodies.

3.8 Recombinant GST-fused protein expression and GST pull down assay

With the exception of GST-SRMS that was procured from SignalChem (#S20-11G, Richmond, BC, Canada), all other GST-tagged constructs were expressed in *E. coli* (BL21 strain), cultured in 2XYT media at 37°C. Protein induction was initiated with the addition of 1 mM IPTG to the bacterial cultures at an optical density of 0.6 at 260 nm wavelength. Bacterial cultures were maintained in a 37°C shaker for 1h. Bacterial cells were then lysed via sonication using short intermittent pulses, in ice-cold 1xPBS buffer containing protease inhibitors: 1 µg/mL aprotinin, and 0.01% phenylmethylsulfonyl fluoride (PMSF), supplemented with a protease inhibitor cocktail comprising 23 mM AEBSF (4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride), 2 mM Bestatin, 100 mM EDTA (Ethylenediaminetetraacetic acid), E-64 0.3 mM trans-epoxysuccinyl-L-leucylamido-(4-guanidino) butane (E 64), 0.3 mM Pepstatin A, in dimethylsulfoxide (DMSO) (P8465, Sigma-Aldrich). Lysates were then incubated with the Glutathione sepharose beads (GST, Novagen) overnight at 4°C on a gyrorotator. The following day, the beads were washed 6-8 times with ice-cold 1x PBS and the beads used for GST- pull down assays.

The pull-down experiments were carried out using GST, GST-SRMS-SH3 and GST-SRMS-SH2 proteins immobilized on glutathione Sepharose beads. The GST-SH3 beads were incubated with HEK293 cell lysates overnight at 4°C on a gyrorotator. Likewise, GST–SH2 beads were incubated with lysates derived from HEK293 cells untransfected or transfected with GFP-SRMS wild-type. Subsequently, the beads were centrifuged, supernatant aspirated and the beads washed 2 times with lysis buffer and 1x PBS. 2x Laemmli buffer was then added to the beads and proteins denatured via incubation at 100°C for 5 minutes before resolving the same via SDS-PAGE.

3.9 *In vitro* kinase assays

In vitro kinase assays were performed using 50 ng GST-SRMS (SignalChem, Richmond, BC, Canada) and 10 µl bed volume of substrate (GST-Dok1 wild type or GST alone) in a reaction volume of 50 µl comprising 20 µl kinase buffer (25 mM MOPS, pH 7.2, 2.5 mM DTT, 12.5 mM and 5 mM EGTA) and 20 µl H_2O with or without 200 µM ATP. The reaction was allowed to proceed by incubating the mixture at 30°C for 30 minutes and was terminated by the addition of 2x Laemmli buffer. The samples were then boiled at 100°C for five minutes and resolved via SDS-PAGE (as described above).

4. Results

4.1 Expression and localization of SRMS in eight human breast cancer cell lines

4.1.1 Relative expression of SRMS in various breast cancer cell lines

While BRK expression has been catalogued in a wide panel of cell lines derived from the normal and tumorigenic human breast, that for SRMS remains undetermined. Therefore, a panel of eight distinct breast cancer cell lines comprising namely, BT-20, MCF7, MDA-MB-231, MDA-MB-435, MDA-MB-468, Au565, HBL-100 and SKBR3 along with a non-tumorigenic mammary epithelial cell line, 184B5 were evaluated for SRMS expression via Western blotting. Since BRK is its closest relative and a known oncogenic protein in breast cancer, SRMS expression was evaluated alongside BRK to facilitate a comparative analysis of the two tyrosine kinase expression profiles. The results indicated a general overexpression of SRMS in six out of eight breast cancer cell lines with HBL-100 exhibiting the highest expression of the protein (Fig 4.1). Still, the expression of SRMS was not absent in any of the cell lines as it was expressed in all eight breast cancer cell lines, albeit low in two of these namely, MDA-MB-468 and AU565 (evidenced upon longer exposure of the X-ray film to the immunoblotted membrane). Interestingly, SRMS expression was also found to be low in the non-tumorigenic mammary epithelial cell line, 184B5. In contrast, BRK expression, while also observed in the majority, was found to be very low or absent in certain cell lines, namely, MDA-MB-435, MDA-MB-468 and HBL-100. The data suggests that (a) SRMS is potentially overexpressed in the majority of breast cancer cell lines and (b) the expression of SRMS appears to be very low in the normal human breast cells while being significantly expressed in the cells derived from breast cancer.

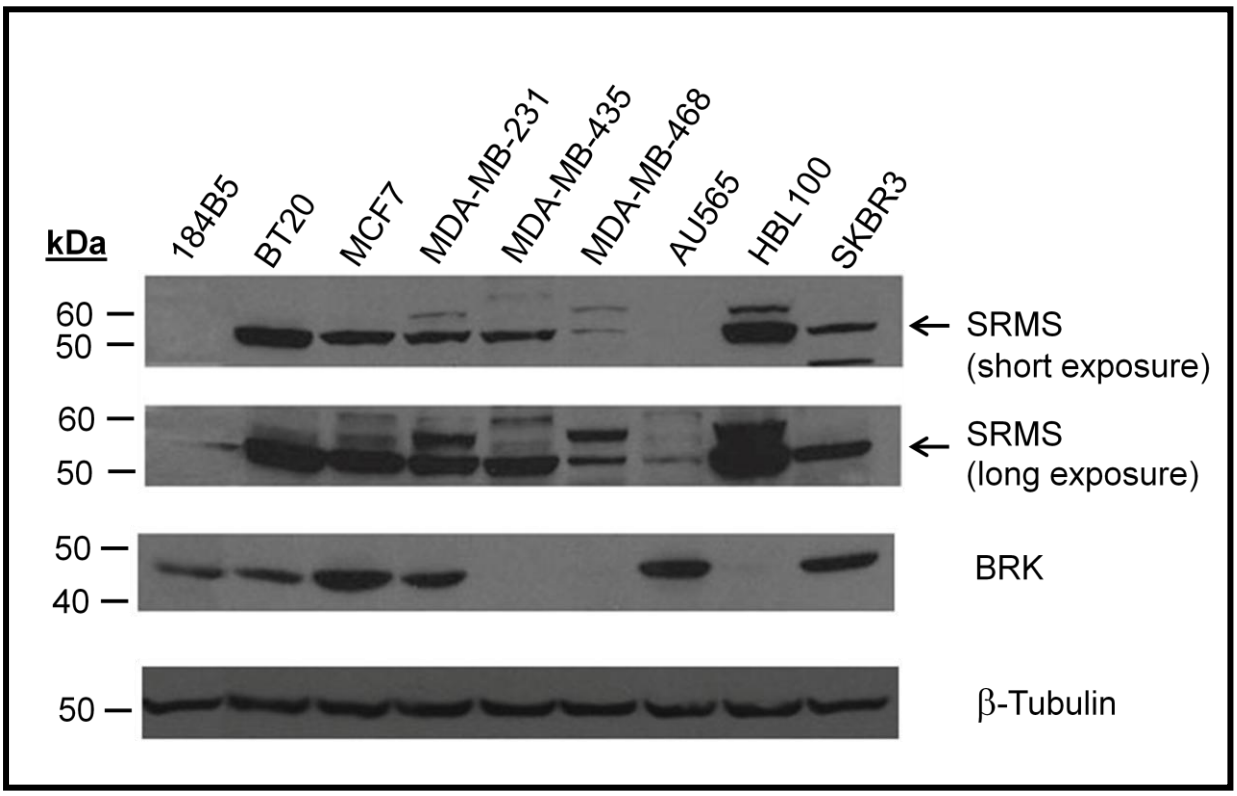

FIGURE 4.1: SRMS is overexpressed in human breast carcinomas. Lysates prepared from breast carcinoma cell lines and a cell line derived from the normal mammary epithelial tissue (184B5), were evaluated for SRMS and BRK expression via immunoblotting with antibodies against SRMS and BRK, respectively. β-tubulin was used as the loading control. The x-ray films were subjected to long and short exposures to determine the expression levels of proteins.

4.1.2 Sub-cellular Distribution of SRMS

The sub-cellular localization of SRMS is unknown and in the absence of a putative myristoylation signal (dictating plasma-membrane localization) as found in Src (Neet and Hunter, 1996) or a characteristic FRK-like nuclear localization signal (directing nuclear localization) (Cance *et al.*, 1994), it was imperative to determine its cellular localization pattern. In order to determine the intracellular localization of the protein, we pursued immunocytochemical analysis of endogenous SRMS in three breast cancer cell lines MDA-MB-231, AU565 and SKBR3 as well as one cervical cancer cell line, HeLa, to determine if the localization varied across different cancer cell lines. Immunofluorescence analysis revealed a predominantly cytoplasmic distribution of endogenous SRMS visible with a distinct punctate pattern in all the cell lines examined (Fig 4.2). No obvious nuclear staining was observed, indicating that SRMS is a predominantly cytoplasmic protein.

Next we wished to compare the endogenous localization pattern with that of ectopically expressed SRMS. GFP-tagged SRMS was transiently transfected in the HEK293 cell line and localization was determined via fluorescence microscopy. Consistent with endogenous SRMS, over-expressed SRMS was also found to localize to punctate regions, which were visible as bright spots in the cytosol of the cells (Fig 4.3).

To further substantiate these results based on the immunofluorescence technique, sub-cellular fractionation was employed in the breast cancer cell lines MDA-MB-231 and AU565 as well as the cervical cancer cell line, HeLa. Indeed, out of the three cellular fractions that were isolated from total cell lysates, endogenous SRMS was found to be exclusive to the cytosolic fraction in all the four cell lines (Fig 4.4).

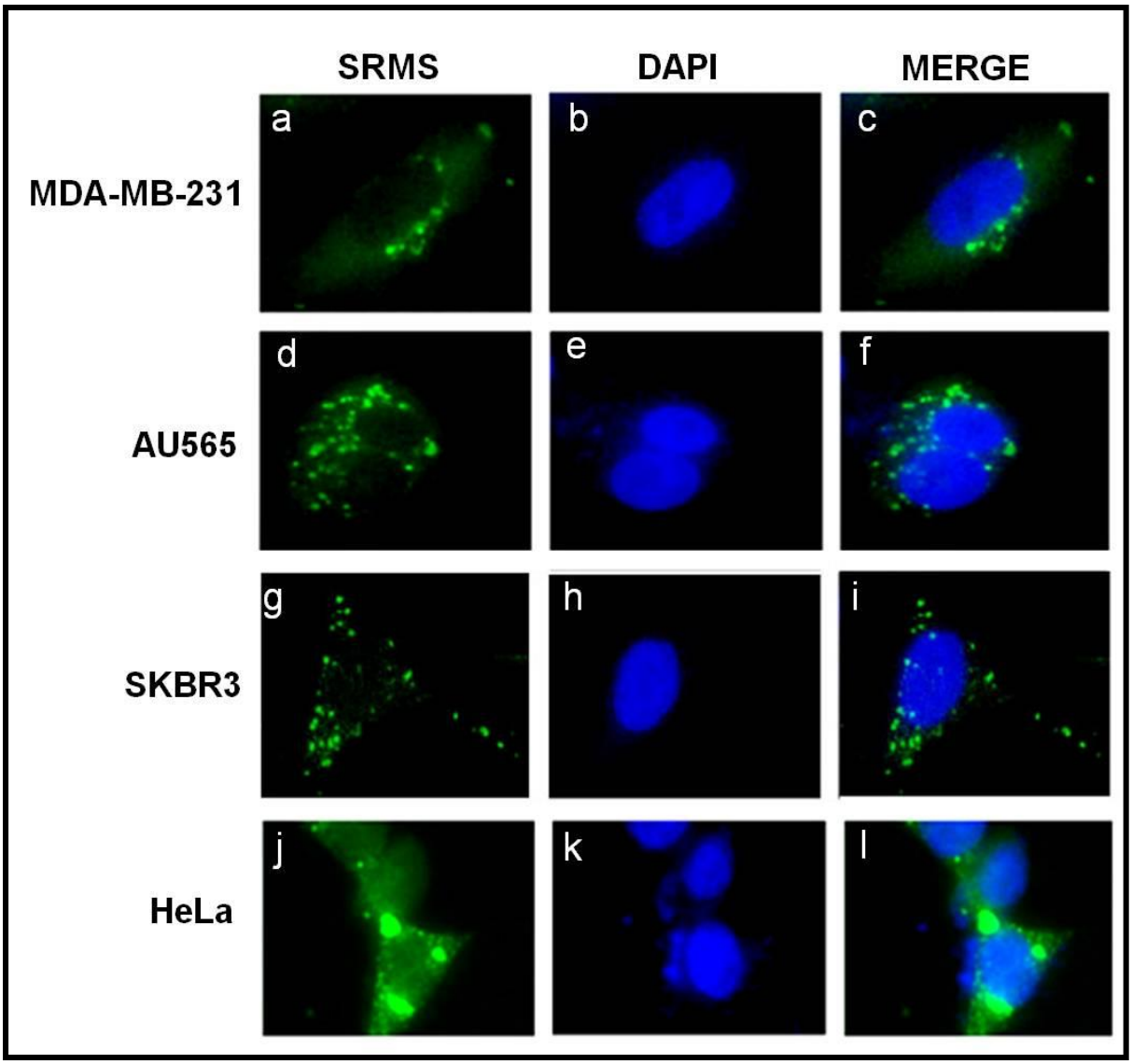

FIGURE 4.2: Endogenous SRMS localizes to punctuate cytoplasmic structures in cells. Intracellular localization of endogenous SRMS was detected via indirect immunofluorecence in the MDA-MB-231 (a-c), AU565 (d-f) and SKBR3 (g-i) human breast cancer cell lines as well as the human cervical cancer cell line, HeLa (j-l). Immunoreactivity was visualized using anti-SRMS and secondary FITC (green) antibodies. Cells were counterstained with DAPI (blue) and images were captured at 60x magnification using the Olympus 1x inverted fluorescence microscope. Similar results were obtained in at least three different experiments.

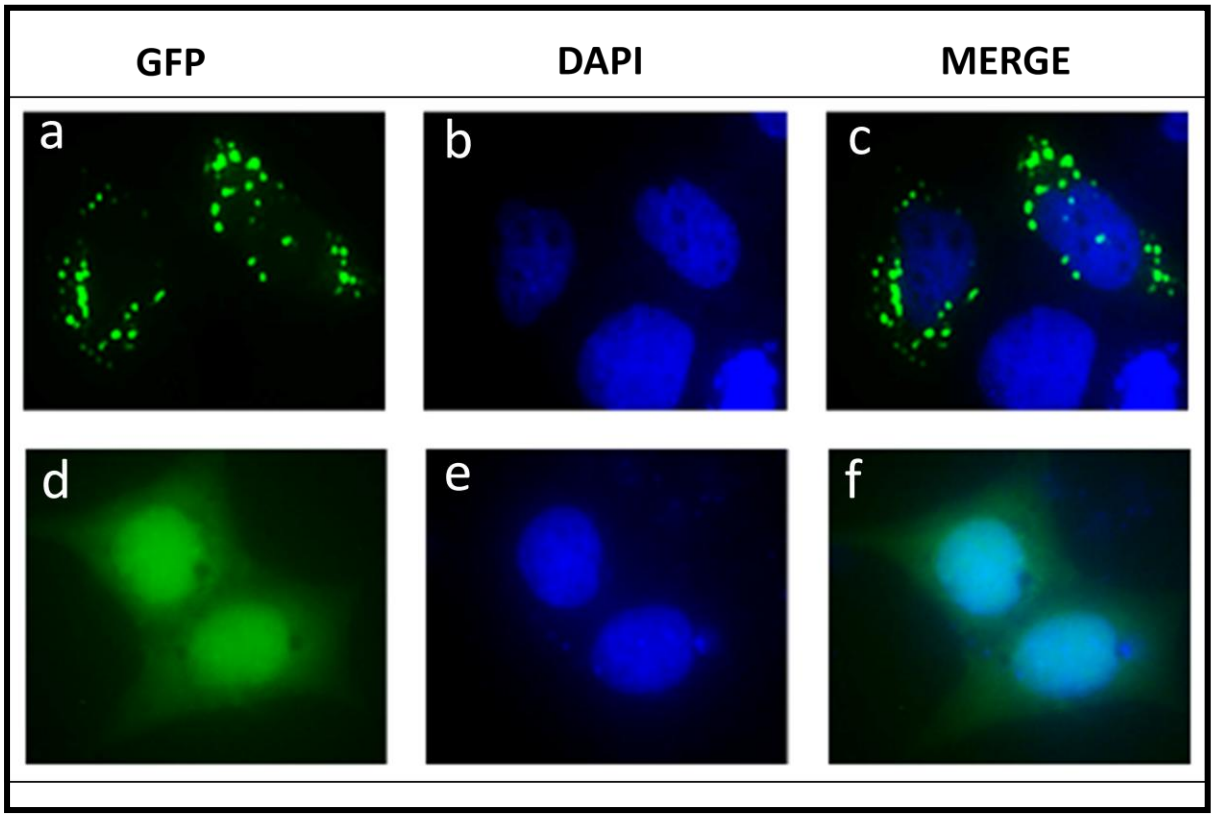

FIGURE 4.3: Ectopically expressed SRMS localizes to punctate cytoplasmic structures in cells. Intracellular localization of exogenous GFP-tagged SRMS (a,b and c) was detected via fluorescence microscopy in the HEK293 cells. pEGFP control vector localization in the same cell line is depicted. (d,e and f). Cells were counterstained with DAPI. Images were captured at 60x magnification using the Olympus 1x51 inverted microscope. Similar results were obtained in at least three different experiments.

\

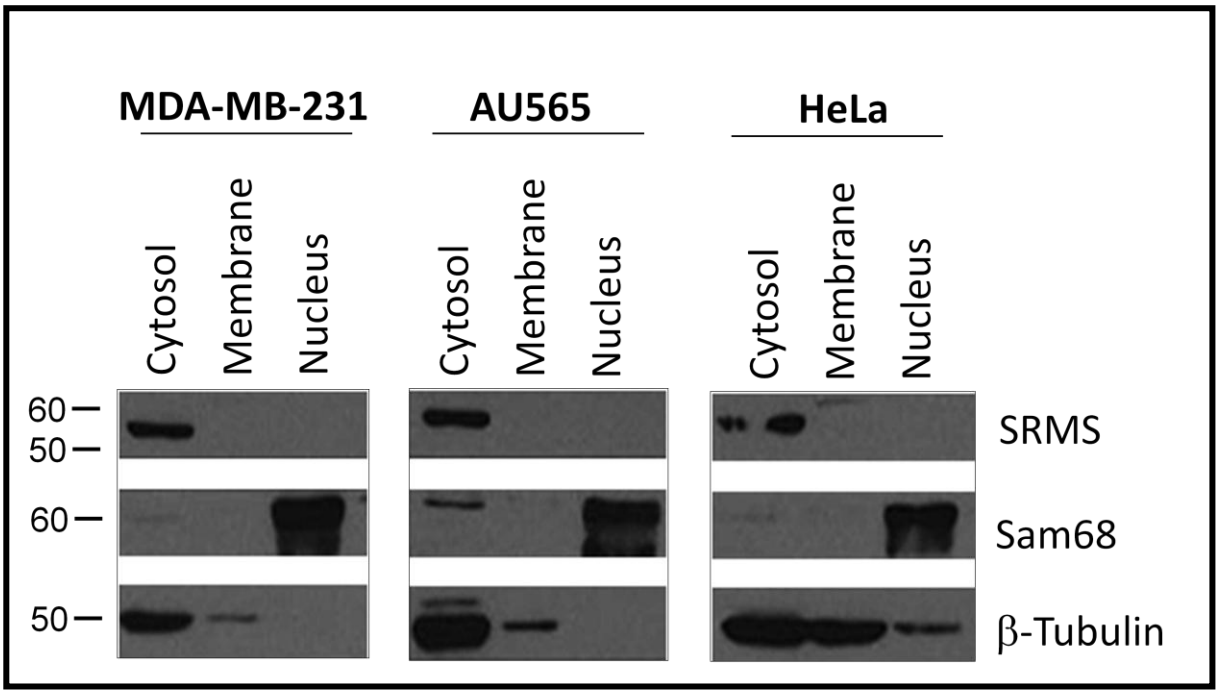

FIGURE 4.4: Sub-cellular fractionation reveals that SRMS is a cytosolic protein. Cells, from the indicated cell lines, were fractionated into the cytosolic, membrane and nuclear fractions and immunoblotted for the detection of SRMS. β-tubulin and Sam68 were used as the controls for the cytosolic/membrane and nuclear compartments, respectively. SRMS is found in the cytosolic fraction of the indicated cell lines. Similar results were obtained in at least three different experiments.

4.2 Biochemical Characterization of SRMS.

4.2.1 Role of the unique N-terminal region of SRMS

Even as SRMS belongs to the same family of non-receptor tyrosine kinases as BRK and exhibits significant structural homology with the latter, there exist subtle variations in their overall protein sequences. One such variation is the absence of a C-terminal region extending beyond the kinase domain in SRMS that is otherwise common to BRK and c-Src (Fig 4.5). The C-terminal region of the latter two PTKs invariably possess a "regulatory" tyrosine, so named for its ability to bind to the respective SH2 domain upon phosphorylation and locking the enzyme in an inactive conformation. SRMS lacks a C-terminal regulatory region but possess an extended and unique N-terminal sequence. In order to understand the functional significance of this N-terminal region and assess the enzymatic activity of SRMS, a truncated mutant of SRMS was generated that lacks the entire N-terminal sequence. Furthermore, utilizing the web-based multiple-sequence alignment tool, CLUSTALW,

certain potentially critical residues in SRMS were identified as conserved with BRK and c-Src. These residues have been previously characterized for their dynamic roles in regulating the catalytic activities of BRK and c-Src. These residues in SRMS correspond to W223, K258 and Y380 (respectively analogous to the functionally diverse: W184 in BRK and W260 in c-Src; ATP-contacting: K219 in BRK and K298 in c-Src and finally, the site for autophosphorylation: Y342 in BRK conserved with Y419 in c-Src). To probe their significance, these residues were mutated independently via site-directed mutagenesis to yield 3 SRMS mutants, namely, SRMS-W223A, SRMS-K219M and SRMS-Y380F.

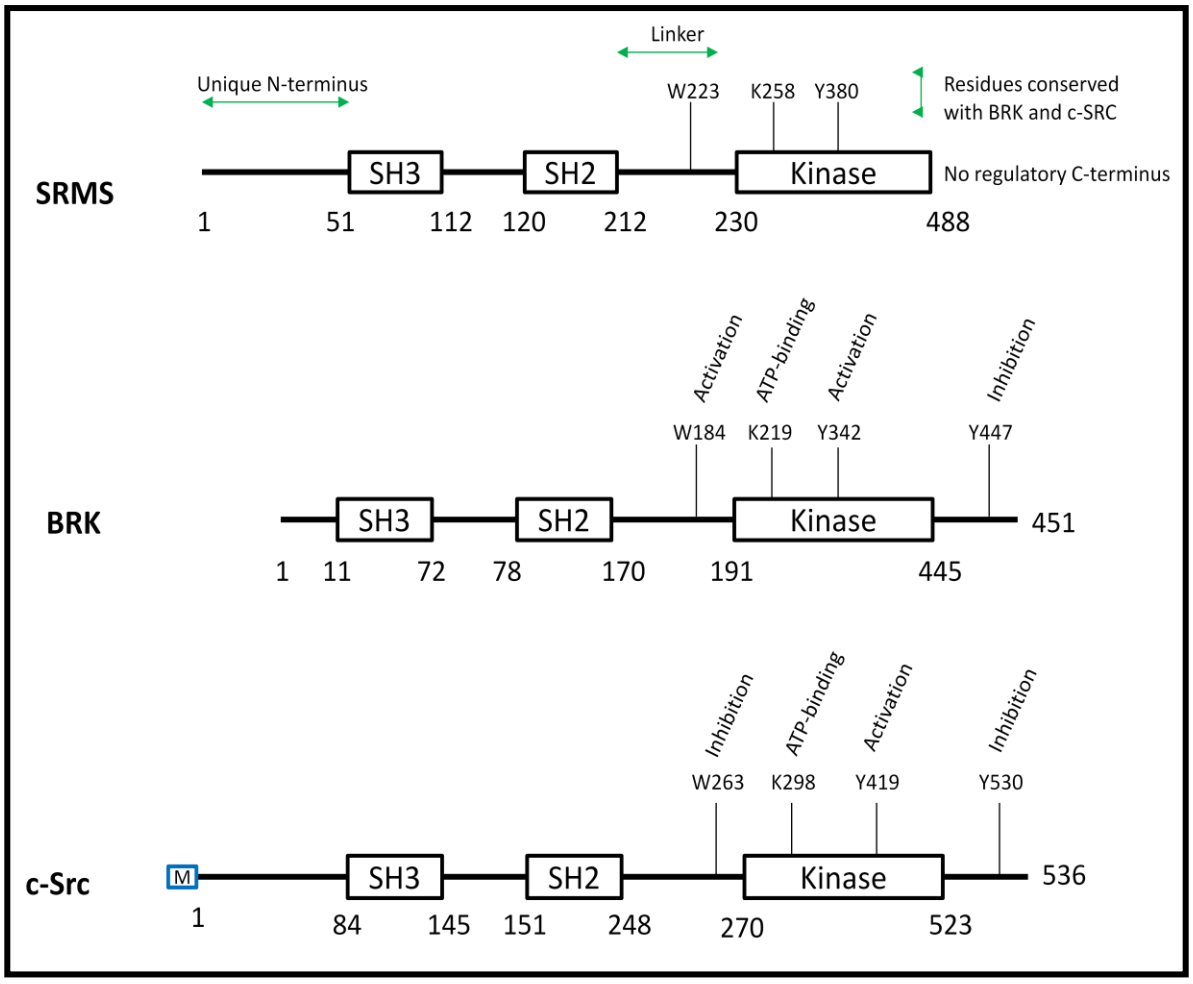

FIGURE 4.5: Schematic representation of SRMS, BRK and c-Src. Shown here are the 3 functional domains, i.e., the SH3, SH2 and the kinase domains, common to the three PTKs. Also shown are the potentially functional residues in SRMS (W223, K258 and Y380) remaining conserved with those that are well characterized in BRK and c-Src. The non-conserved N-terminal region of SRMS and the myristoylation signal in c-Src (represented by "M") are also depicted.

To assess the outcome of such mutations, including ΔN-SRMS, on the overall catalytic activity of SRMS, the GFP-tagged mutants (GFP-SRMS-K258M and GFP-SRMS-W223A), along with wild-type SRMS, were transiently transfected in HEK293 cells. As well, to draw comparative analysis, GFP-tagged BRK wild-type as well as its kinase-dead (GFP-BRK-K219M) and constitutively-active (GFP-BRK-Y447F) mutants were also transiently transfected in the same cell line. Immunoblotting was performed using anti-GFP and anti-phosphotyrosine (pY20) antibodies to determine, respectively, the levels of the overexpressed proteins and the relative levels of tyrosine phosphorylation via evaluating total phosphorylation of endogenous targets.

Firstly, it was noted, from anti-phosphotyrosine immunoblotting, that wild-type SRMS exhibits strong intrinsic tyrosine kinase activity that is almost comparable to that of the wild-type BRK, but not to that of its hyper-active mutant, BRK-Y447F (Fig 4.6). Secondly, it was also noted that the deletion of the 51 amino acid N-terminal segment (ΔN-SRMS), preceding the SH3 domain of SRMS totally abolishes the enzymatic activity. Thirdly, an anticipated absence of kinase activity with the K258M mutant was also observed since interfering with the ATP-contact site is expected to abolish the kinase's ability to acquire and utilize the phosphate group derived from ATP for phosphorylation (Qiu and Miller, 2004). This result validates the SRMS K258 as a critical residue of the ATP-binding site within the PTK's kinase domain, akin to K219 and K298 of BRK and c-Src, respectively. However, mutating the conserved tryptophan (W223) within the SH2-kinase linker, also resulted in a sharp decrease in the overall tyrosine kinase activity. This was particularly interesting since an analogous mutation in the Src family kinase, Hck, (W260) caused an increase in its kinase activity (via SH3-dependent kinase inhibition) (LaFevre-Bernt *et al.*, 1998). The outcome in SRMS was similar to that in BRK wherein a significantly decreased kinase activity was observed upon mutation of the conserved tryptophan W184 (Qiu and Miller, 2004).

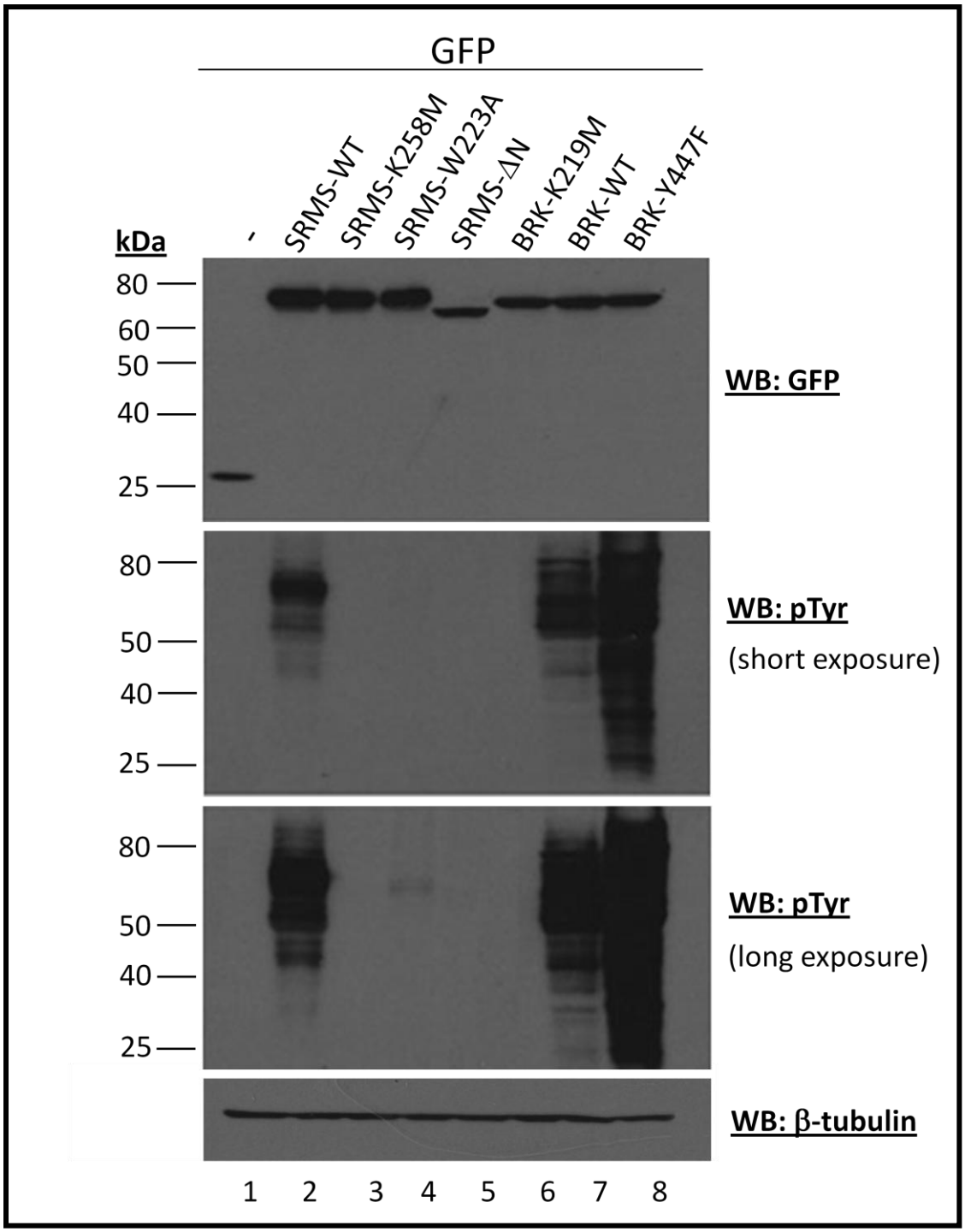

FIGURE 4.6: The N-terminal region of SRMS regulates its kinase activity. Lysates prepared from HEK-293 cells transfected with the indicated SRMS and BRK constructs, were evaluated to determine the relative kinase activity of the variants using antibodies against total phosphotyrosines (bottom panel). Expression of the ectopic proteins was probed via anti GFP (Top panel). β-tubulin was used as the loading control. Similar results were obtained in at least three different experiments.

Autophosphorylation is a measure of enzyme activation as studied in Src family kinases as well as in BRK. In order to determine whether the levels of total tyrosine phosphorylation observed in Figure 4.6 corresponded to the autophosphorylation levels of the enzymes, wild-type SRMS as well as its three mutants, ΔN, W223A and Y380F, were transiently transfected in HEK293 cells. The overexpressed proteins were then immunoprecipitated from the cell lysates using anti-GFP antibodies and analysed by immunoblotting with anti-GFP and anti-phosphotyrosine antibodies. As shown in Fig. 4.7, while wild-type SRMS and BRK displayed prominent autophosphorylation, the SRMS-Y380F exhibited markedly reduced autophosphorylation, thereby validating that this tyrosine residue is indeed the site for autophosphorylation in SRMS. Furthermore, upon longer exposure of the X-ray film, a subtle degree of autophosphorylation was noticed in the W223A mutant, whereas ΔN-SRMS displayed no detectable autophosphorylation.

From investigations on a small cohort of mutations introduced in the SRMS protein sequence (Fig 4.6 and Fig 4.7), it was evident that the linker region W223, the ATP-contacting K258, the autophosphorylation site Y380 and the N-terminus segment are involved in the enzyme activity. Yet the involvement of other potential regulatory elements in SRMS was unknown. Therefore, the study was extended to include additional SRMS mutants (as described below and shown in Fig 4.8, 4.9 and 4.10). Catalytic regulation via the SH3 and SH2 domains has been documented for Src family kinases as well as BRK (Qiu and Miller, 2004). In order to understand the role of these domains in regulating enzymatic activity of SRMS, GFP-tagged mutants comprising deletions of either the SH3 domain (ΔSH3 SRMS) or the SH2 domain (ΔSH2 SRMS) were generated.

In Src family kinases, there exists a highly conserved arginine residue that forms part of the "signature FLVRES motif" within the SH2 domain and contacts the target phosphotyrosine. Mutation of the conserved R175 in Src has been shown to destabilize phosphotyrosine binding (Campbell and Jackson, 2003). Also, mutation of a similar arginine in BRK (R105) resulted in reduced phosphorylation and binding to the BRK substrate, STAP2 (Mitchell *et al.*, 2000). Nonetheless, while the ΔSH2 mutant of BRK exhibits higher kinase activity, the effect of the R105 mutation has not been reported on the enzyme's catalytic activity (Qiu and Miller, 2004). Sequence alignment using CLUSTALW revealed that SRMS also possesses an arginine (R147) that remains conserved with BRK and c-Src.

Arginine 147 in SRMS was thus mutated to alanine to assess the catalytic repercussions alongside its ΔSH2 mutant.

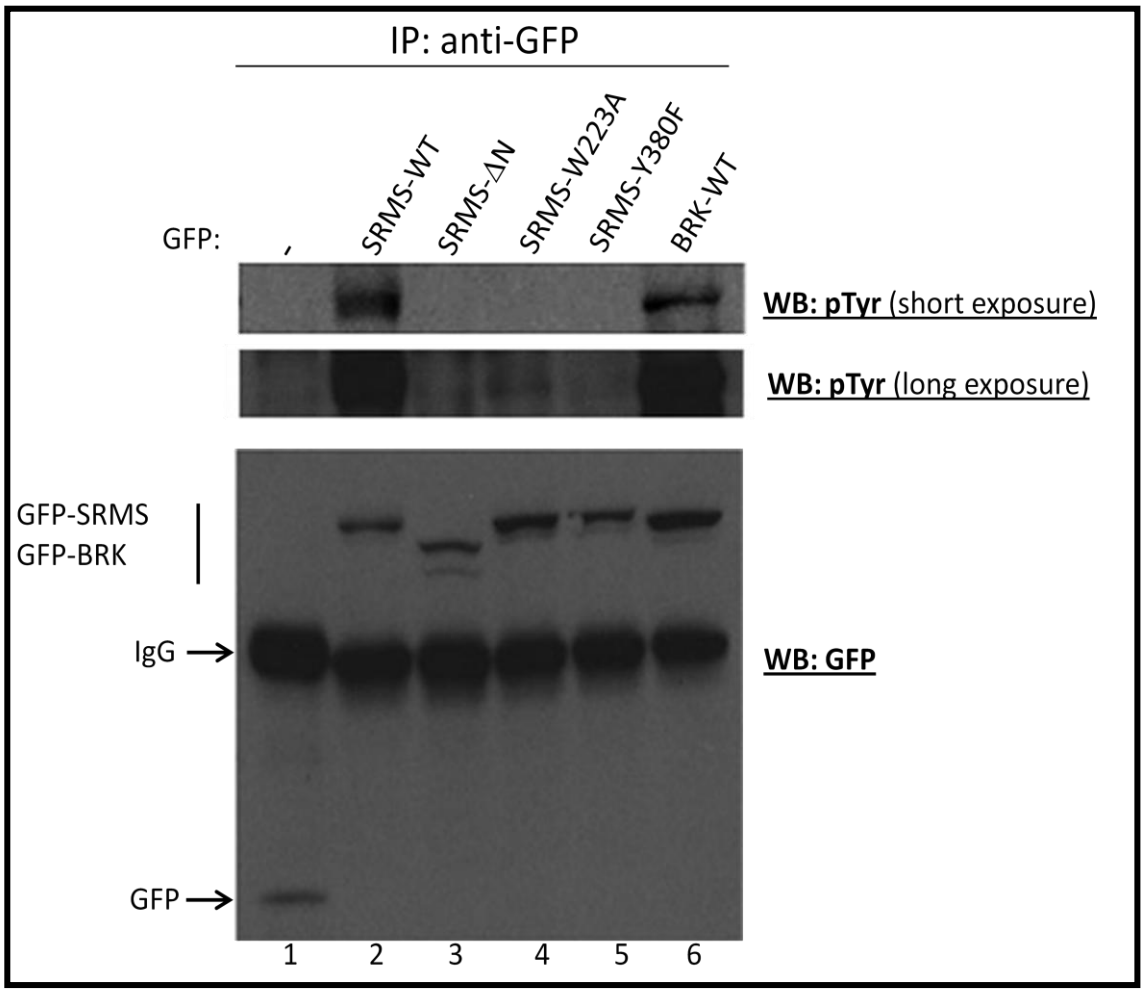

FIGURE 4.7: SRMS is autophosphorylated on Y380. Wild-type BRK and wild-type SRMS as well as its indicated mutants were immunoprecipitated from HEK-293 cell lysates and immunoblotted with anti-phosphotyrosine (top panel) to reveal autophosphorylation of the respective proteins. The immunoprecipitated proteins were probed via anti GFP (bottom panel). . Similar results were obtained in at least three different experiments.

From the results in Figure 4.6 and 4.7, it was demonstrated that the N-terminal region plays a critical role in stabilizing the intrinsic tyrosine kinase activity of SRMS. The primary amino acid sequence of this N-terminal region shows that this region is replete with proline residues. In fact, out of the total 50 amino- acids constituting the N-terminal region, nine were prolines. Furthermore, six such prolines, located proximal to the SH3 domain, were found to be closely

stacked in a manner that was indicative of a discrete proline-rich motif. This small proline-rich segment was therefore deleted and the resulting mutant was designated PΔN-SRMS (where "P" stands for Partial).

The SH2-kinase linker region of BRK contains three proline residues, conforming to the PXXP motif that participates in intra-molecular interactions with the SH3 domain, thus facilitating the inactive state of the PTK (Qiu and Miller, 2003). The SRMS SH2-kinase linker region also possesses three invariable proline residues, albeit not conforming to a PXXP type motif. Two different mutants were constructed taking into account the spatial arrangement of these prolines. In one mutant, a single proline situated proximal to the SH2 domain was mutated to alanine (P214A) and the mutant designated SRMS-PA. The other variant contained mutations in the remaining two prolines (P218A+P226A) located distally from the SH2 domain and was designated SRMS-2PA

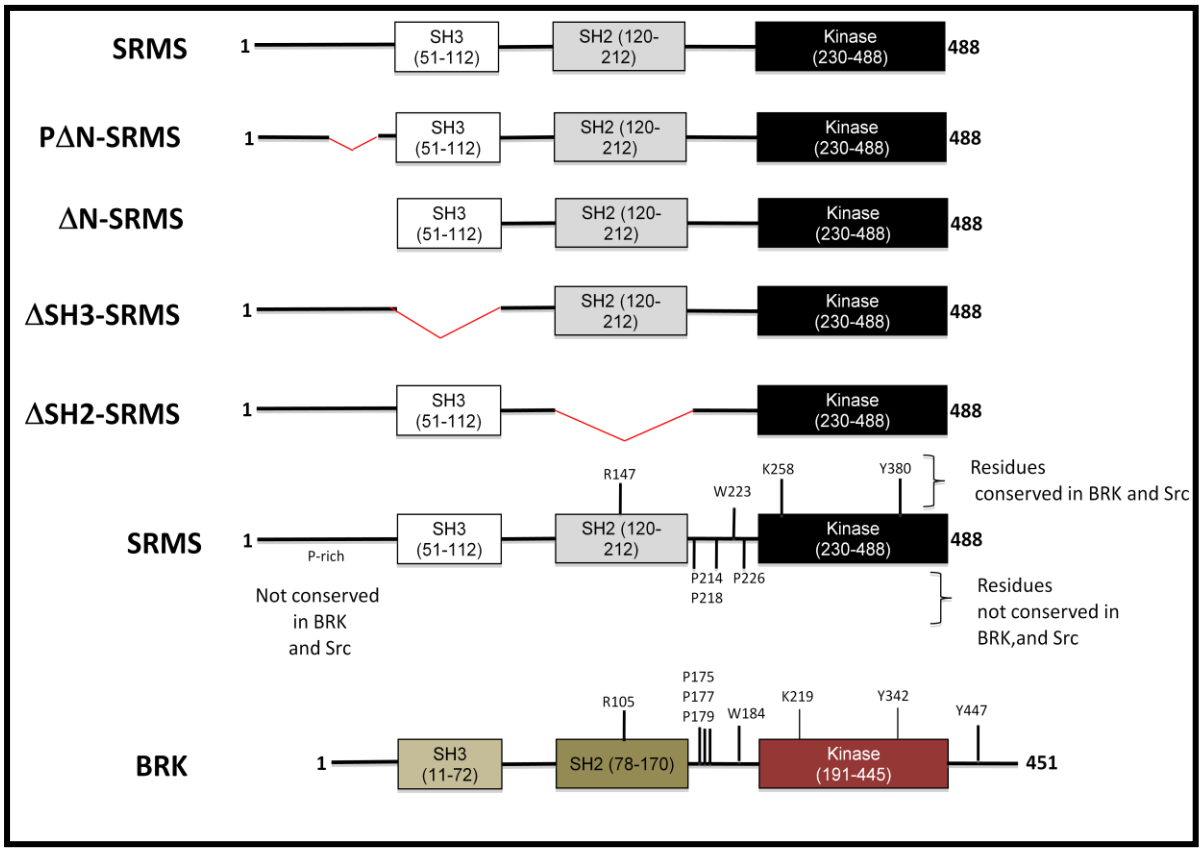

FIGURE 4.8: Schematic representation of SRMS mutants. Shown here is the wild type SRMS containing the globular functional domains SH3, SH2 and the kinase domain. SRMS mutants; ΔN-SRMS (N-terminally deleted), PΔN-SRMS (Partial N-terminal deletion), ΔSH3 (SH3 deletion), ΔSH2 (SH2 deletion) as well as its point mutated variants for the indicated

amino acid residues are also shown. The critical residues W223A, K258, Y380 and R147 are conserved with BRK.

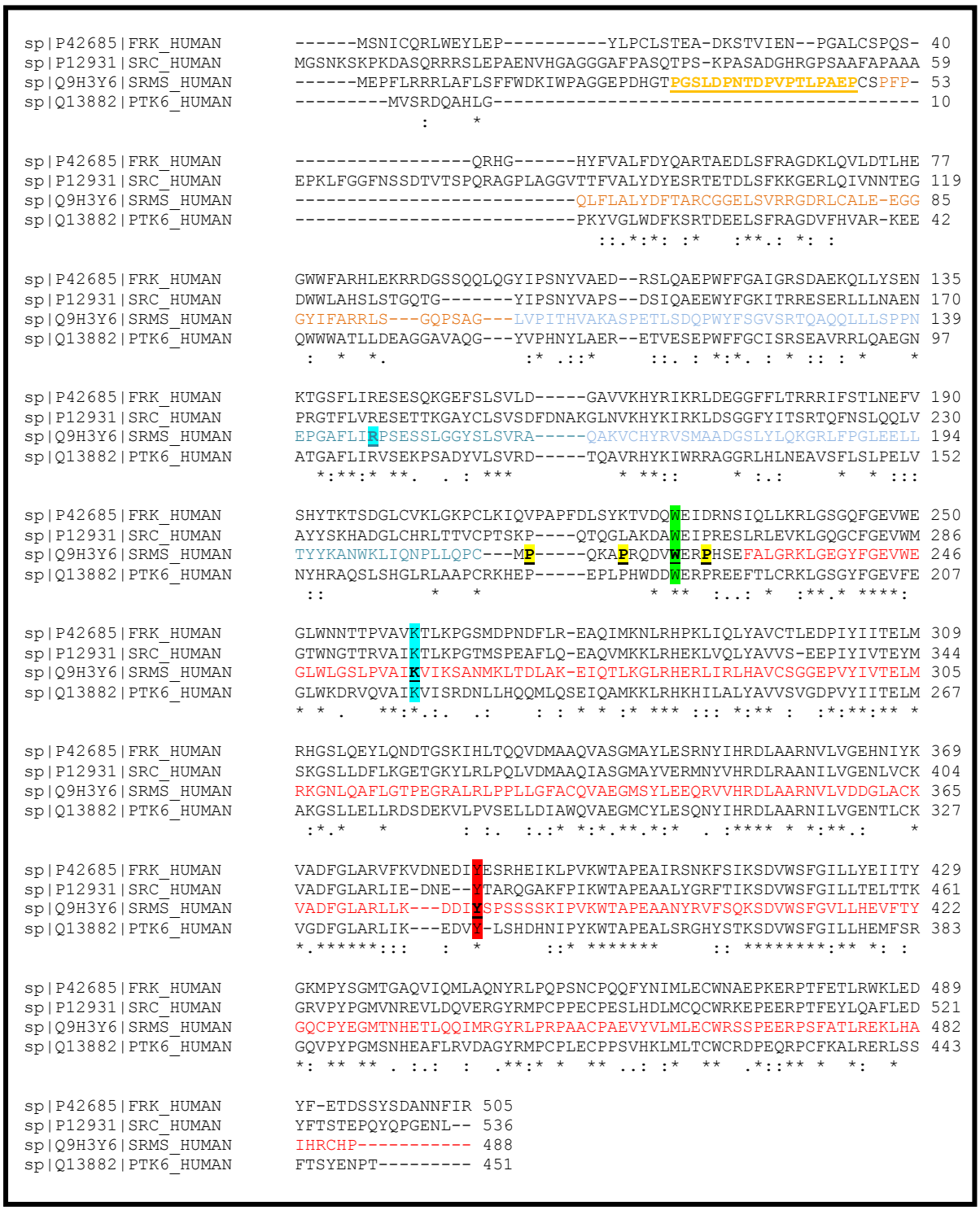

FIGURE 4.9: **Multiple sequence alignment of c-Src and FRK/PTK6 family kinases**. FRK, BRK and SRMS with c-Src depicting their autophosphorylated tyrosines (red), ATP-binding lysines (dark green) and auto-regulatory tryptophans (light green). Other amino acids selected for point-mutations in SRMS are highlighted: P214 and P218+P226 (yellow)

and R147 (light grey). A short proline-rich segment at the N-terminus of SRMS (highlighted and underlined in gold) was deleted to generate the PΔN SRMS mutant. The SRMS SH3 domain (51-112) amino acids are in orange, the SH2 domain (120-212) in blue and the kinase domain (230-488) in red. "*" represent conserved amino acid residues across the given amino acid sequences. ":/." represent partially conserved amino acid residues.

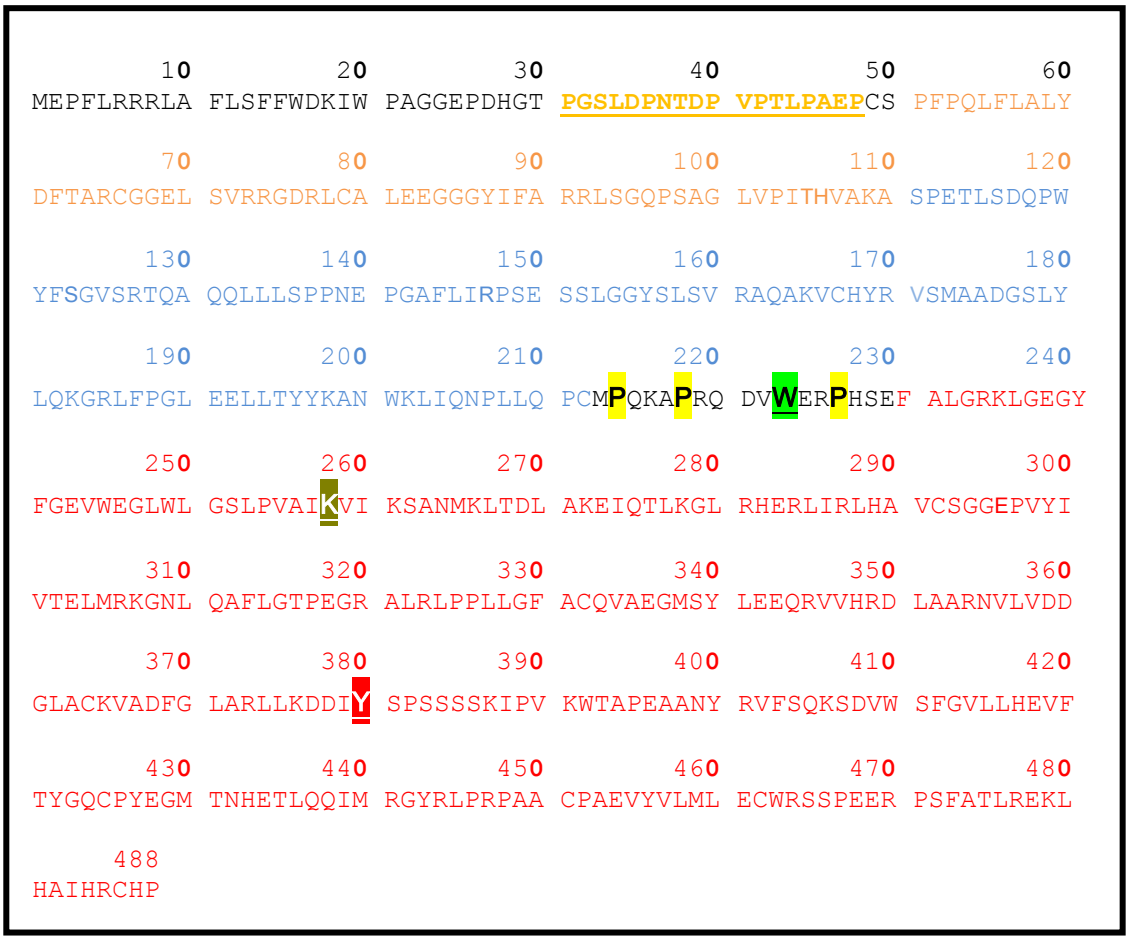

FIGURE 4.10: **Amino acid sequence of SRMS highlighting the various residues that were mutated.** These include P214A, P218A+P223A, W223A, K258 and Y380F. The short N-terminal proline-rich segment (highlighted and underlined in gold) was deleted to generate the PΔN SRMS mutant. The amino acids comprising the SH3 domain (51-112) are in orange, those comprising the SH2 domain (120-212) are in blue and those for the kinase.

All of these mutants together with wild-type SRMS, ΔN-SRMS, SRMS-K258M and SRMS-W223A were transiently transfected in HEK293 cells. Whole cell lysates were utilized for immunoblotting with antibodies against GFP, SRMS and phosphotyrosines. All these mutants together with wild-type SRMS, ΔN-SRMS, SRMS-K258M and SRMS-W223A were transiently transfected in HEK293 cells to test the overall effects on kinase activity. Cell lysates were analyzed for immunoreactivity with anti-phosphotyrosine antibodies to assess the relative kinase activity (Fig. 4.11, panel A and B). The variants were generally expressed at comparable levels (Fig. 4.11, panel C and D). Note that since anti-SRMS targets an epitope spanning the N-terminal region of the protein, the detection of the PΔN SRMS was reduced while that of ΔN SRMS was significantly decreased. Of all the SRMS mutants examined, ΔSH2-SRMS (lane 7) displayed the lowest kinase activity compared with the wild-type SRMS, suggesting that SH2 domain is essential for SRMS kinase activation. However, deletion of the SH3 domain did not significantly affect kinase activity (lane 8). P214A (lane 4) and 2PA (lane 5) mutations in the linker region also had little effect on kinase activity. As well, the R147A mutantdisplayed kinase activity that was similar to that of the wild-type SRMS (compare lane 1 and lane 6). Also, the PΔN-SRMS failed to alter the catalytic activity of SRMS, suggesting that the short segment rich in proline residues does not display a critical role towards regulating SRMS tyrosine kinase activity. Taken together, our data demonstrates that the kinase activity of SRMS is regulated by its unique N-terminal sequence. Furthermore, contrarily to BRK (Qiu and Miller, 2004), deletion of the SH2 domain of SRMS resulted in a significantly reduced kinase activity. Overall, these data demonstrate that the catalytic activity of SRMS is regulated by its unique N-terminus sequence as well as its SH2 domain.

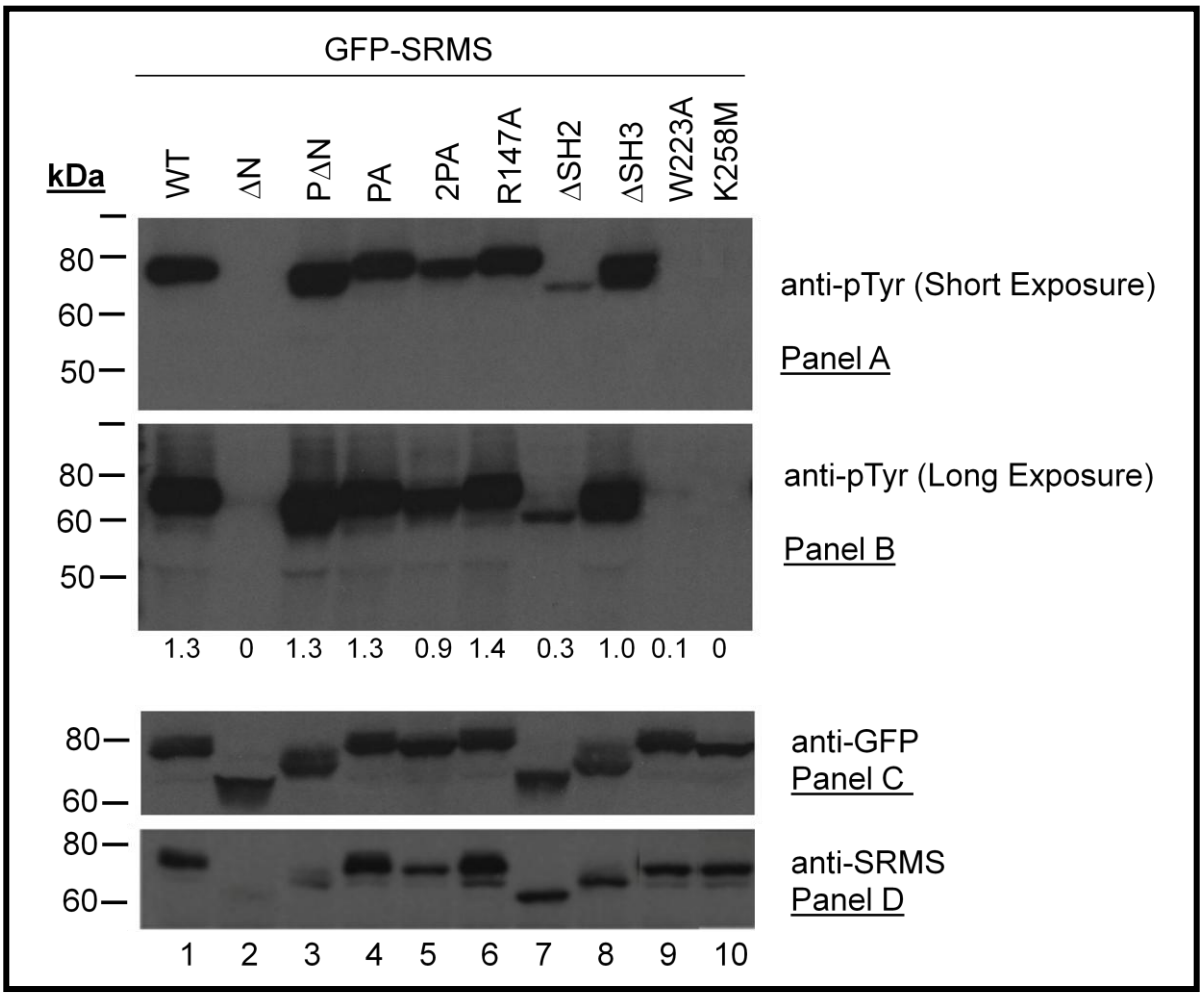

FIGURE 4.11: The N-terminal region of SRMS and its SH2 domain promotes a constitutively active form of the kinase. Transiently transfected variants of SRMS were analysed for relative catalytic activity in comparison to wild type SRMS via immunoblotting with phosphotyrosine antibodies (panel A and B). Anti-GFP was used to probe the ectopically expressed proteins (panel C). Anti-SRMS antibodies, targeting an epitope in the N-terminus region of SRMS, depict commensurately abrogated immunoreactivity with ΔN-SRMS and PΔN-SRMS (panel D). Protein bands were quantified using the IMAGE J software (1.24) and expressed in terms of fold increase relative to the controls (anti-GFP). Similar results were obtained in at least three different experiments.

4.2.2 Sub-cellular localization of SRMS mutants

From the data in Figure 4.3 and 4.4 it was shown that endogenous as well as overexpressed SRMS exhibits a punctate cytoplasmic sub-cellular distribution pattern. The cellular mechanisms regulating such a characteristic localization pattern are unknown. SRMS has functional SH3 and SH2 domains that mediate intermolecular interactions with cellular targets. It is therefore conceivable that SRMS is directed to specific sub-cellular compartments through its SH3 and/or SH2 mediated interactions with endogenous protein targets. It is also possible that the unique N-terminal region of SRMS regulates not only kinase activity but also sub-cellular localization. To this end, wild type SRMS as well as its mutants SRMS-K258M, SRMS-Y380F, SRMS-W223A, ΔSH3-SRMS, ΔSH2-SRMS and ΔN-SRMS were transiently transfected in HEK293 cells and localization analysed through fluorescence microscopy. We observed that while wild-type SRMS localized to punctate cytoplasmic structures in over 90% of transfected cells, the SRMS-K258M mutant displayed a diffuse pattern in approximately 80% of transfected cells (Fig. 4.12). However, the SRMS-W223A as well as the SRMS-Y380F mutants exhibited a predominant punctate sub-cellular distribution comparable to wild-type SRMS (Fig 4.12 and 4.14). Interestingly, while the ΔSH3 mutant produced a punctate pattern in approximately 80% of transfected cells, ΔSH2-SRMS as well as ΔN-SRMS exhibited a diffuse localization pattern in roughly 90% and 70% of the transfected cell population, respectively (Fig 4.13 and 4.14). Together, these data suggest that the N-terminal region of SRMS not only regulates the enzyme's activity, but can also modulate the sub-cellular localization. These findings also indicate that the SH2 domain of SRMS may contribute to sequester SRMS in specific cytoplasmic structures.

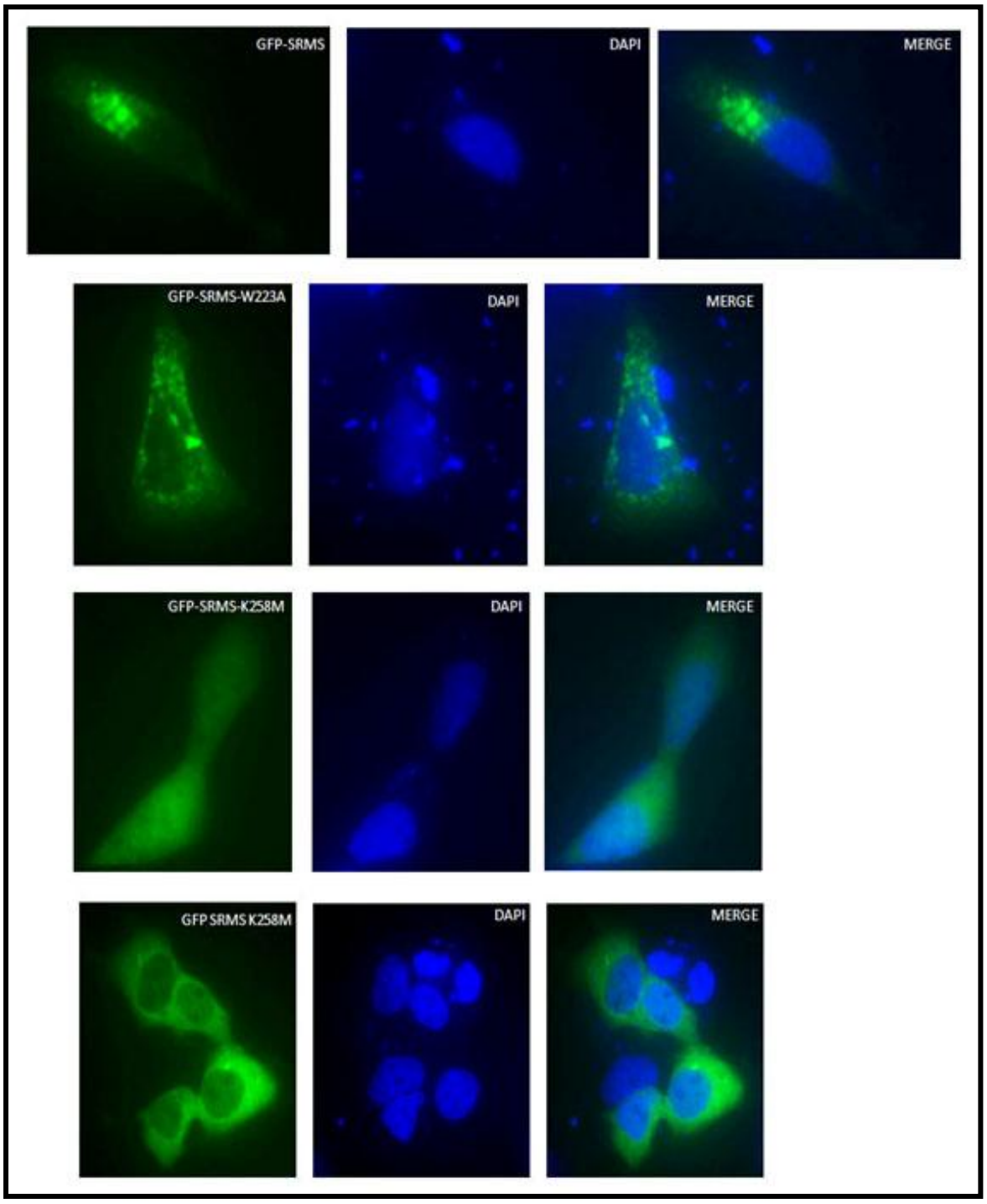

FIGURE 4.12: Sub-cellular localization of wild type SRMS and its mutants; SRMS-K258M and SRMS-W223A. Intracellular localization of exogenous GFP-tagged wild-type SRMS and its indicated mutants, was detected via fluorescence microscopy in HEK293 cells. Cells were counter-stained with DAPI. Images were captured at 60x magnification using the Olympus inverted microscope. Similar results were obtained in at least three different experiments.

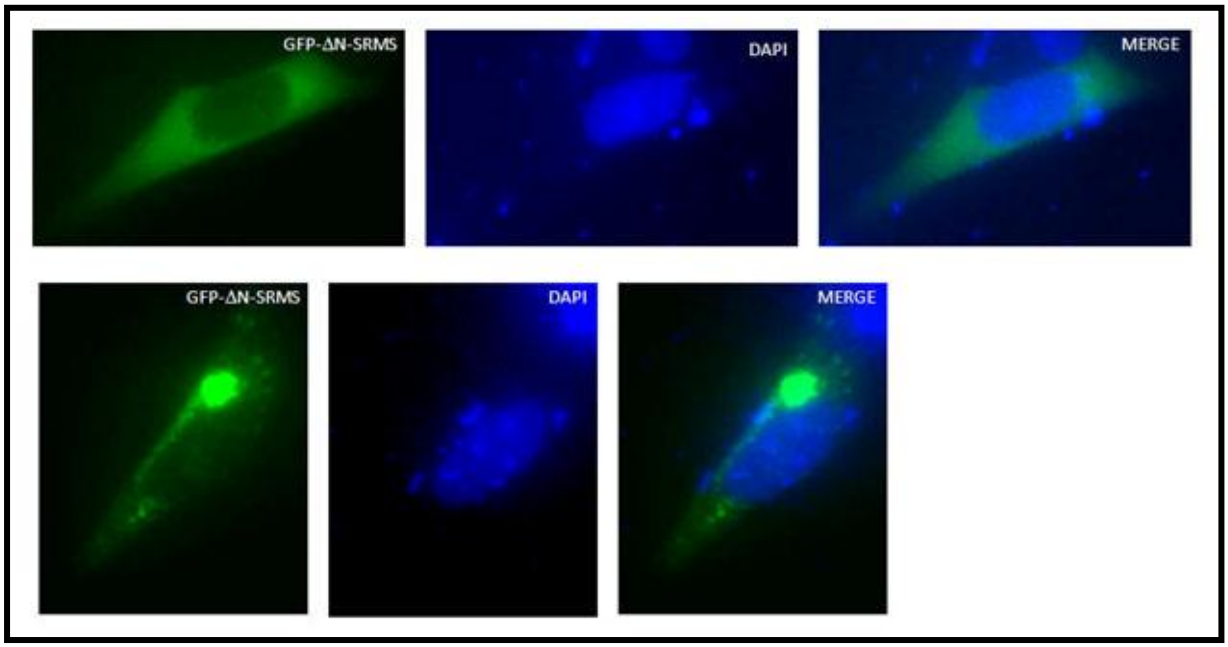

FIGURE 4.13: Sub-cellular localization of ΔN SRMS. GFP-ΔN SRMS was transiently transfected in HEK293 cells. Cells were counter-stained with DAPI. Images were captured at 60x magnification using the Olympus inverted microscope. Similar results were obtained in at least three different experiments.

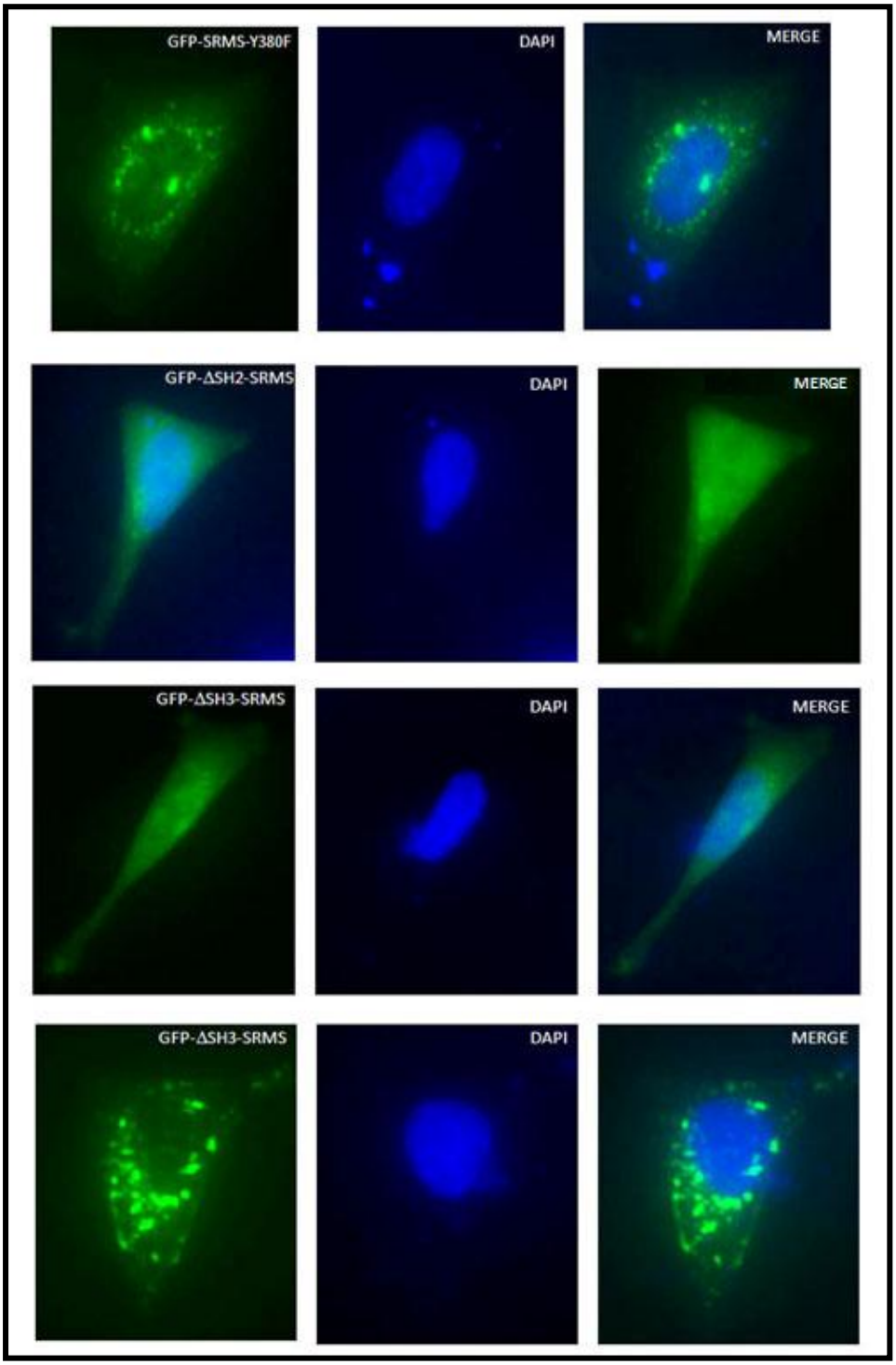

FIGURE 4.14: Sub-cellular localization of ΔSH2-SRMS, ΔSH3-SRMS and Y380F-SRMS. SRMS mutants, GFP-SRMS-Y380F, GFP-ΔSH2-SRMS and GFP-ΔSH3-SRMS were transiently transfected in HEK293 cells. Cells were counter-stained with DAPI. Images were captured at 60x magnification using the Olympus inverted microscope. Similar results were obtained in at least three different experiments.

4.3 Expression and Sub-cellular localization of Dok1 in various breast cancer cell lines.

4.3.1 Expression of Dok1 in breast cancer cell lines

Although Dok1 has been validated as a tumor suppressor in several studies, there is little information on its expression in cancer cell lines, particularly those derived from the human breast, prompting us to investigate its expression in these cell lines. Endogenous Dok1 expression was determined and compared alongside SRMS in eight breast cancer cell lines, namely BT20, MCF7, MDA-MB-231, MDA-MB-435, MDA-MB-468, AU565, HBL-100 and SKBR3, besides the non-tumorigenic breast epithelial cell line, 184B5. Interestingly, though Dok1 was found to be low or absent in four cell lines, namely, MCF7, MDA-MB-468, AU565 and SK-BR3, its elevated expression in the other cell lines, especially in HBL-100, appeared to correspond with that of endogenous SRMS (Fig 4.15).

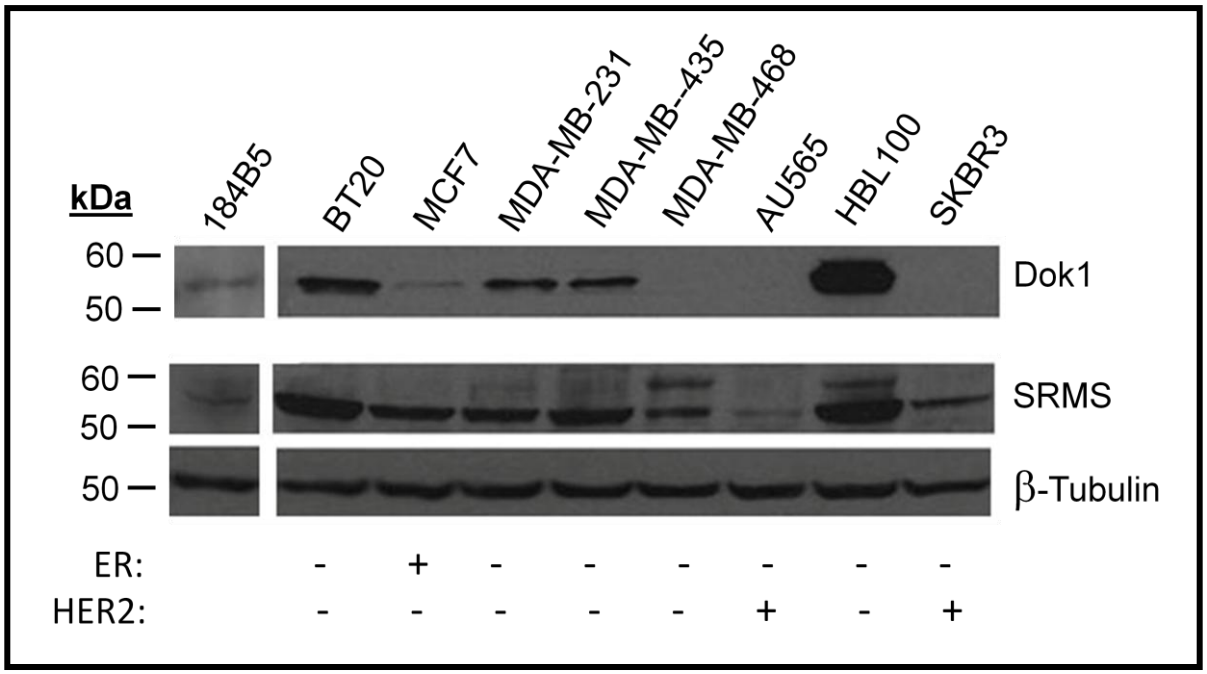

FIGURE 4.15: Dok1 is differentially expressed in breast cancer cell lines. Dok1 expression was surveyed alongside SRMS in 8 breast cancer cell lines and a normal breast epithelial cell line, 184B5, using antibodies against Dok1 and SRMS, respectively. β-tubulin was used as the loading control. Similar results were obtained in at least three different experiments.

4.3.2 Sub-cellular distribution of Dok1

Through immunocytochemistry analysis using anti-Dok1 antibodies, the sub-cellular localization of endogenous Dok1 was determined in the MDA-MB-231 and MDA-MB-435 cell lines as well as in the HeLa cell line. Based on such immunofluorescence analysis, endogenous Dok1 was found to localize predominantly to the cytosol of the cell lines (Fig 4.16). Like endogenous Dok1, ectopically expressed GFP-Dok1, was also found to localize to the cytoplasm, as analysed through fluorescence microscopy, in HEK293 cells (Fig 4.17). Sub-cellular fractionation studies further corroborated data from immunocytochemistry analysis (Fig 4.18).

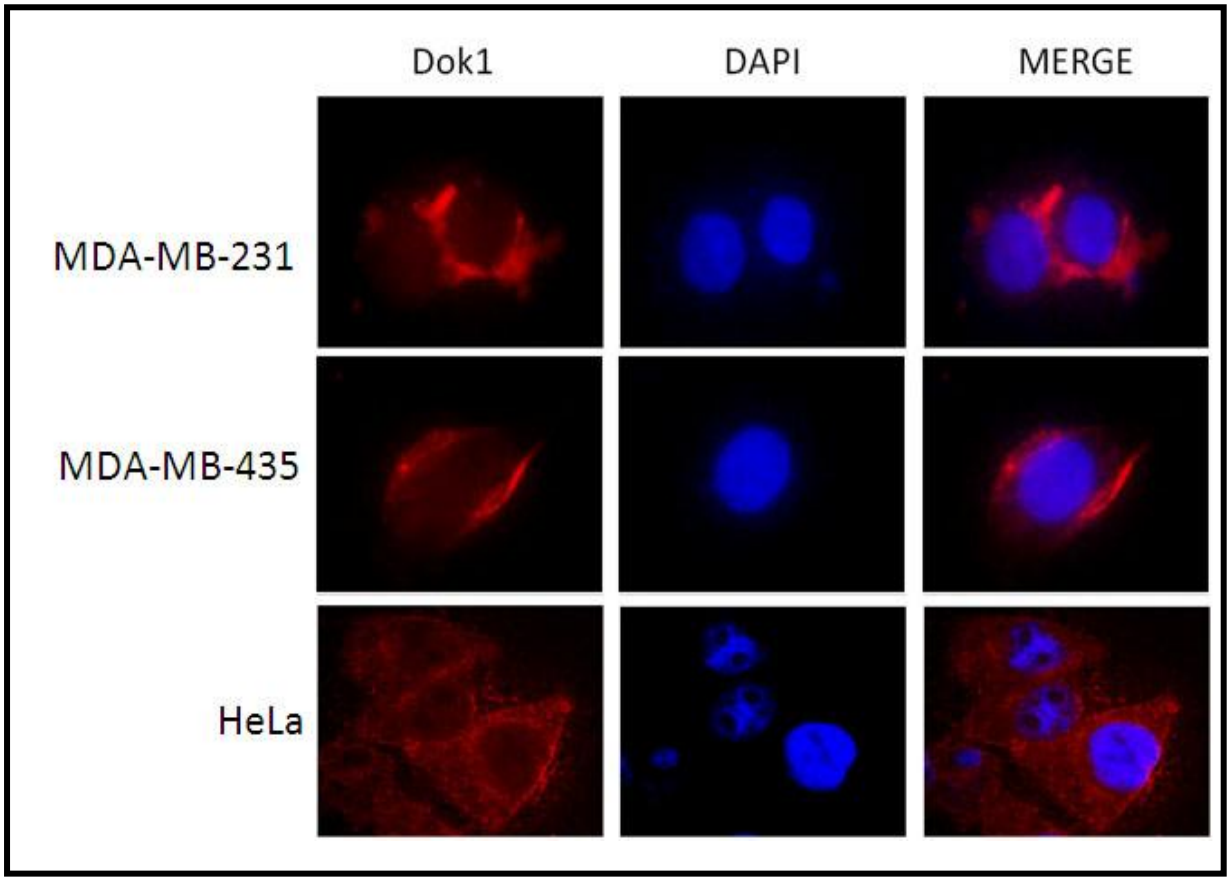

FIGURE 4.16: Endogenous Dok1 localizes predominantly to the cytosol in cells. A. Intracellular localization of endogenous Dok1 was detected via indirect immunofluorecence in the breast cancer cell lines, MDA-MB-231 and MDA-MB-435 and the cervical cancer cell line, HeLa. Immunoreactivity was visualized using anti-Dok1 and secondary TEXAS RED. Cells were counterstained with DAPI (blue). Similar results were obtained in at least three different experiments.

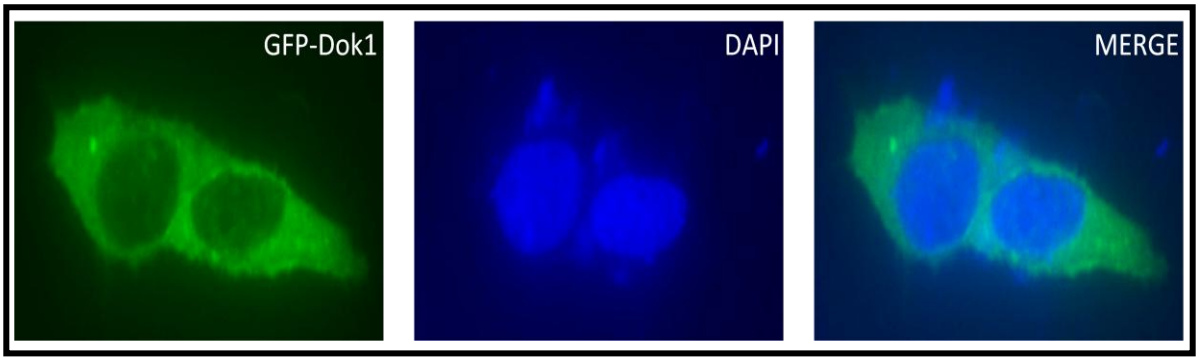

FIGURE 4.17: Ectopically expressed Dok1 localizes predominantly to the cytosol in cells. HEK293 cells were transiently transfected with GFP-Dok1 and localization determined via fluorescence microscopy. Cells were counter-stained with DAPI and images taken at 60x magnification using the Olympus inverted microscope. Similar results were obtained in at least three different experiments).

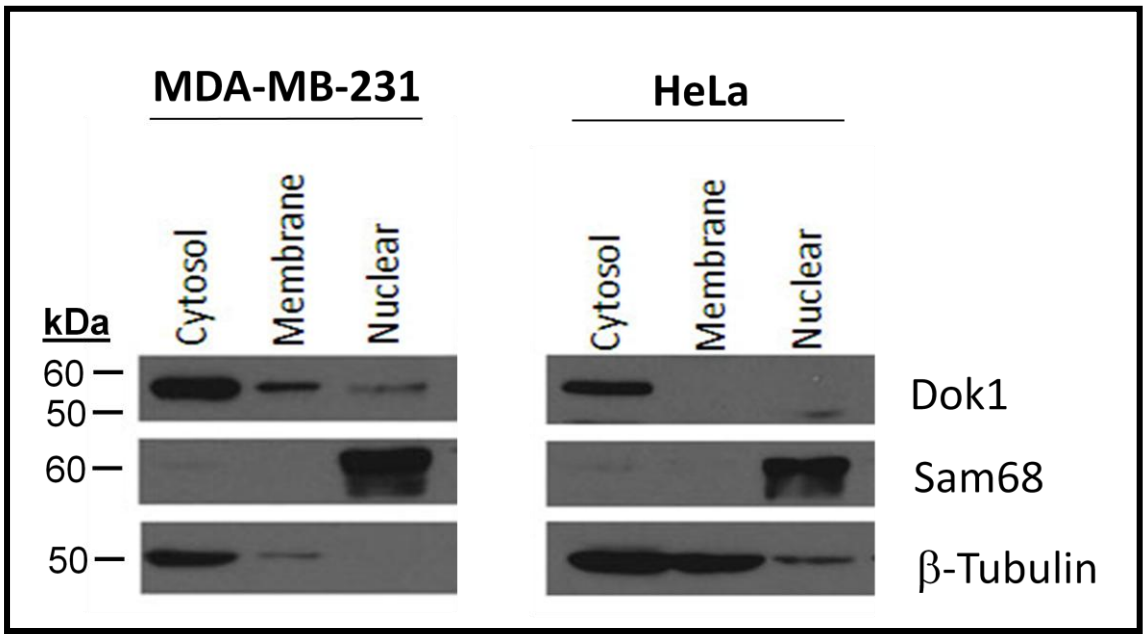

FIGURE 4.18: Sub-cellular fractionation reveals that Dok1 is a predominantly cytoplasmic protein. Cells from the indicated cell lines were fractionated into the cytosolic, membrane and nuclear fractions and immunoblotted for Dok1. β-tubulin and Sam68 were used as the fraction markers for the cytosolic/membrane and nuclear compartments, respectively. Similar results were obtained in at least three different experiments.

Since Dok1, akin to SRMS, was found to localize to the cytoplasm, it was next determined whether both proteins co-localized in cells. Using antibodies specific to SRMS and Dok1, minimal co-localization was observed between endogenous Dok1 and SRMS in the HEK293 cell line (Fig 4.20).

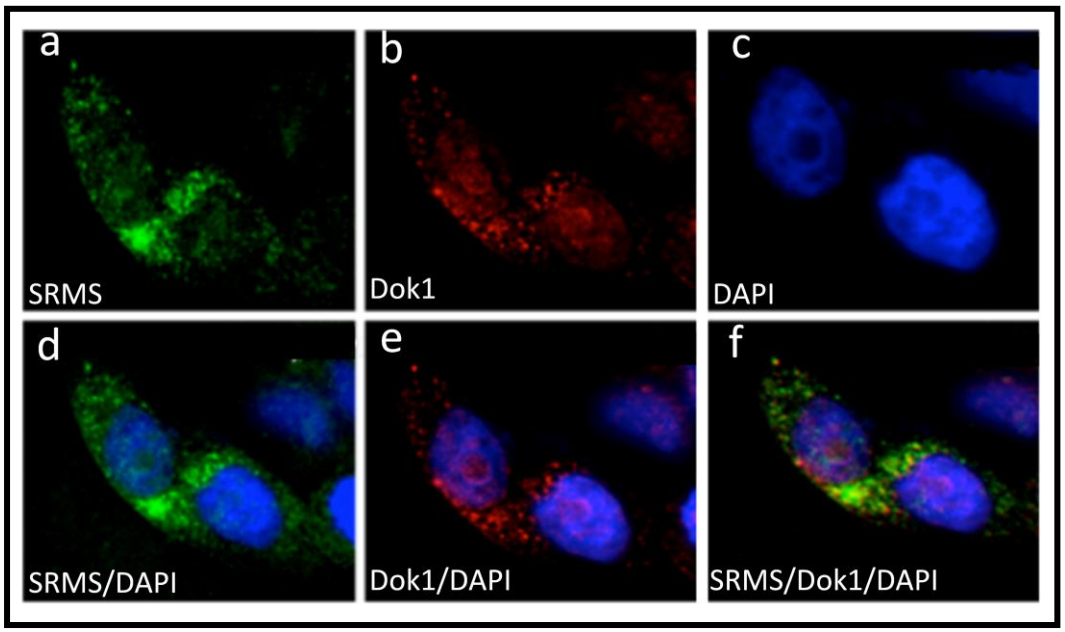

FIGURE 4.19: Co-localization analyses of endogenous Dok1 and SRMS. Endogenous SRMS and Dok1 co-localization was determined via indirect immunofluorescence in the HEK293 cell line using antibodies against SRMS (FITC) and Dok1 (Texas Red). Cells were counterstained with DAPI. Images were taken at 60x magnification using the Olympus 1x51 inverted microscope. Similar results were obtained in at least three different experiments (n=3).

4.4 Characterizing Dok1 as a substrate of SRMS

4.4.1 Molecular interaction between SRMS and Dok1

SRMS has SH3 and SH2 domains which in Src family kinases as well as in BRK and FRK have been shown to bind to proline-rich sequences and phosphorylated tyrosine residues respectively. The C-terminal tail of Dok1 possesses several proline and tyrosine residues that may serve as potential SH3/SH2 binding sites (Fig 1.11).

Since Dok1 was identified as a potential target of SRMS, we first determined whether both proteins interact. To study binding associations between both proteins, GFP-SRMS and

GFP-Dok1 were transiently transfected either alone or together in HEK293 cells. The cells were then lysed and subjected to immunoprecipitation with antibodies against Dok1 and SRMS. Immunoblotting was performed using the same antibodies to determine co-immunoprecipitation of SRMS with Dok1 and vice-versa. Immunoblotting the Dok1 immunoprecipitates with anti-SRMS antibodies revealed the co-precipitation of SRMS with Dok1 (Fig 4.20, lane 7). Reciprocal co-precipitation of Dok1 was also observed in SRMS immunoprecipitates (Fig 4.20, lane 7).

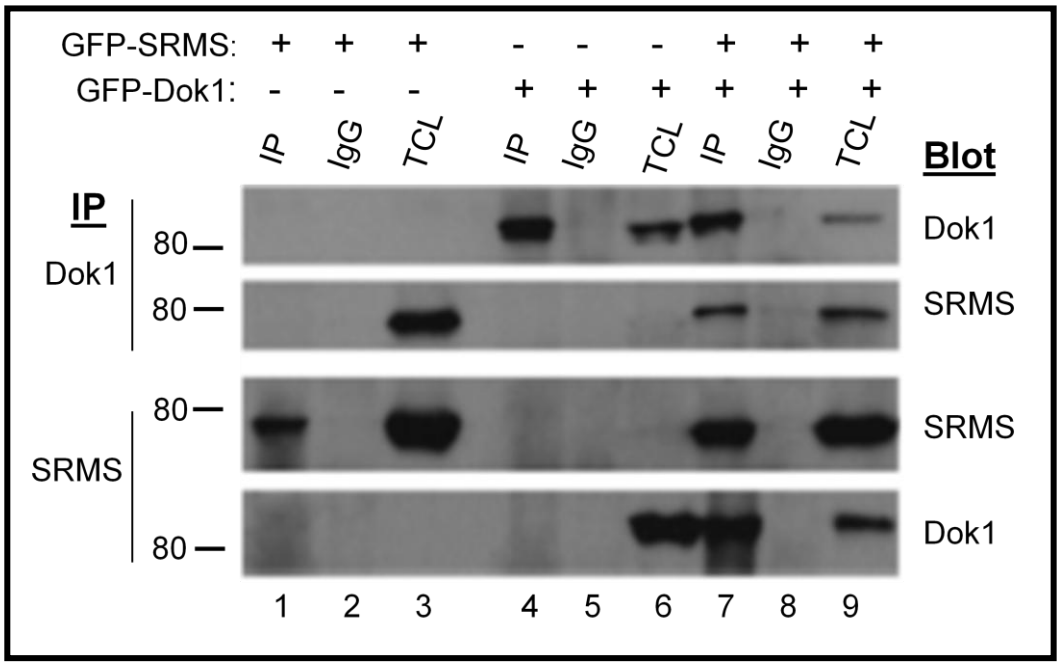

FIGURE 4.20: SRMS interacts with Dok1 in cells. HEK293 cell lysates from GFP-SRMS, GFP-Dok1 or GFP-SRMS/GFP-Dok1 co-transfected cohorts were subjected to immunoprecipitation with anti-Dok1 and immunoblotted with Dok1 and SRMS (top 2 panels). Conversely, SRMS was immunoprecipitated from such lysates using anti-SRMS and the immunoprecipitates (IP) probed for SRMS and Dok1 (bottom 2 panels). Anti-IgG (Rabbit) was used as the control. Total cell lysates (TCL) indicate the relative expression of the proteins. Similar results were obtained in at least three different experiments.

To ensure that the association between the GFP-tagged SRMS and Dok1 proteins was not due to GFP dimerization, HEK293 cells transiently transfected with either pEGFP control vector or co-transfected with pEGFP and GFP-Dok1 or GFP-Dok1 and ΔN-SRMS, were subjected to immunoprecipitation with anti-GFP antibodies and immunoblotted with the same antibody. As can be seen in Fig 4.21, GFP alone did not co-precipitate with GFP-Dok1 (Lane 4) whereas GFP-ΔN-SRMS was found to co-precipitate with GFP-Dok1 (Lane 7). Thus, the

results in Figure 4.21 demonstrate that (a) the association between the proteins is not via GFP dimerization and (b) Dok1 binds to ΔN-SRMS. These results clearly suggest that SRMS physically associates with Dok1 and that the N-terminal region of SRMS, while being indispensible for catalytic activity, is potentially expendable for binding associations of the enzyme with its targets.

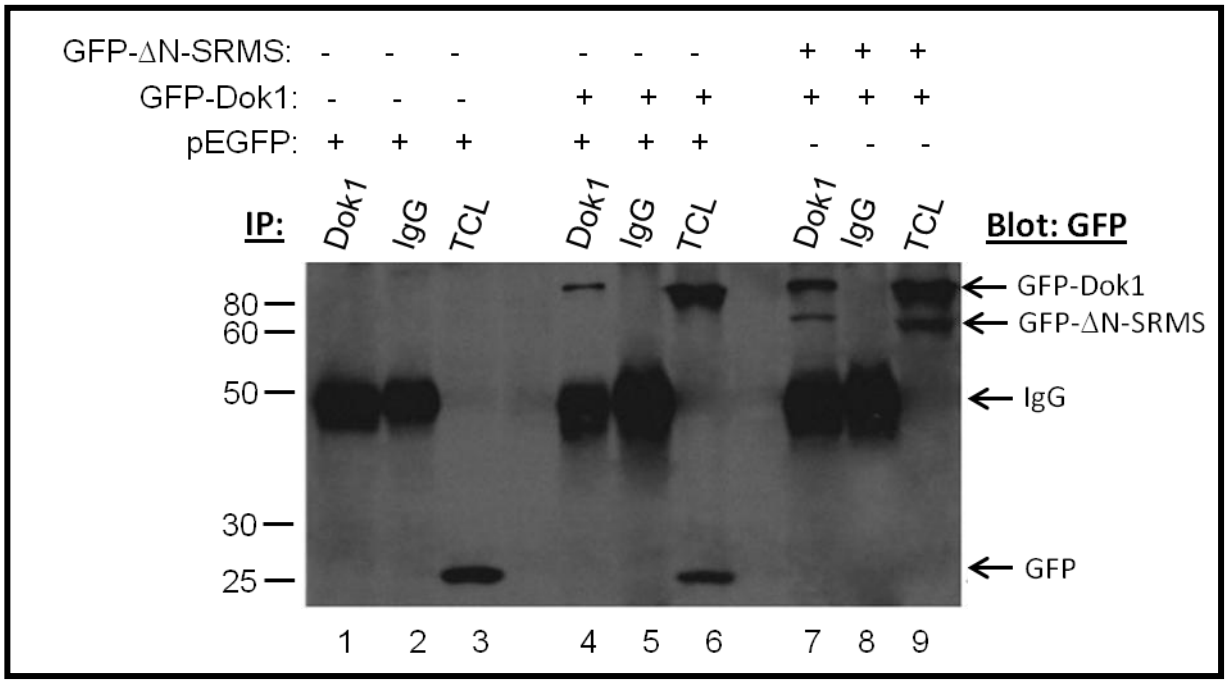

FIGURE 4.21: The association between GFP-SRMS and GFP-Dok1 is not mediated via GFP dimerization. Cell lysates were subjected to immunoprecipitation with anti-Dok1 in the GFP-Dok1/pEGFP or GFP-Dok1/GFP-ΔN SRMS co-transfected cohorts and immunoblotted with antibodies against GFP to probe for GFP dimerization-mediated association between the ectopic proteins. Anti-IgG (Rabbit) was used as the control. Total cell lysates (TCL) indicate the relative expression of the proteins. Similar results were obtained in at least three different experiments.

To determine whether SRMS associates with Dok1 via its SH3 and/or SH2 domains, *in vitro* binding assays were performed using the glutathione S-transferase (GST)-fused SH3 and SH2 domains of SRMS. First, the association between endogenous Dok1 and GST-SH3 was evaluated using lysates from untransfected HEK293 cells. Using antibodies against Dok1, interactions between GST-SH3 and Dok1 were observed (Fig. 4.22, left panel**)**. However, such binding asociations were not observed with the GST-SH2 protein. Since the SH2 domain interacts primarily with phosphorylated tyrosine residues, HEK293 cells were

transiently transfected with GFP-SRMS to determine whether ectopically expressed SRMS will induce the phosphorylation of endogenous Dok1 and its binding to the SH2 domain of SRMS. As shown in Fig 4.23 (left panel), GST-SH2 formed a complex with endogenous DOK1 in the presence of GFP-SRMS. Moreover, immunoblotting with anti-phosphotyrosine antibodies revealed the bound Dok1 to be tyrosine phosphorylated. Such binding interactions between GST-SH2 and Dok1 was not observed in the presence of pEGFP vector or the kinase-dead mutant of SRMS, SRMS-K258M, thus demonstrating that tyrosine phosphorylation of Dok1 is required for associations with the SRMS SH2 domain (Fig 4.24). Expression and molecular sizes of the GST, GST-SH3 and GST-SH2 proteins are shown by Coomassie Blue staining (Fig 4.22 and 4.23, right panels). GST alone bound to beads was used as control and total cell HEK293 lysates were used to determine the relative expression of endogenous Dok1. These data collectively show that both, the SH3 and SH2, domains of SRMS mediate interactions with Dok1.

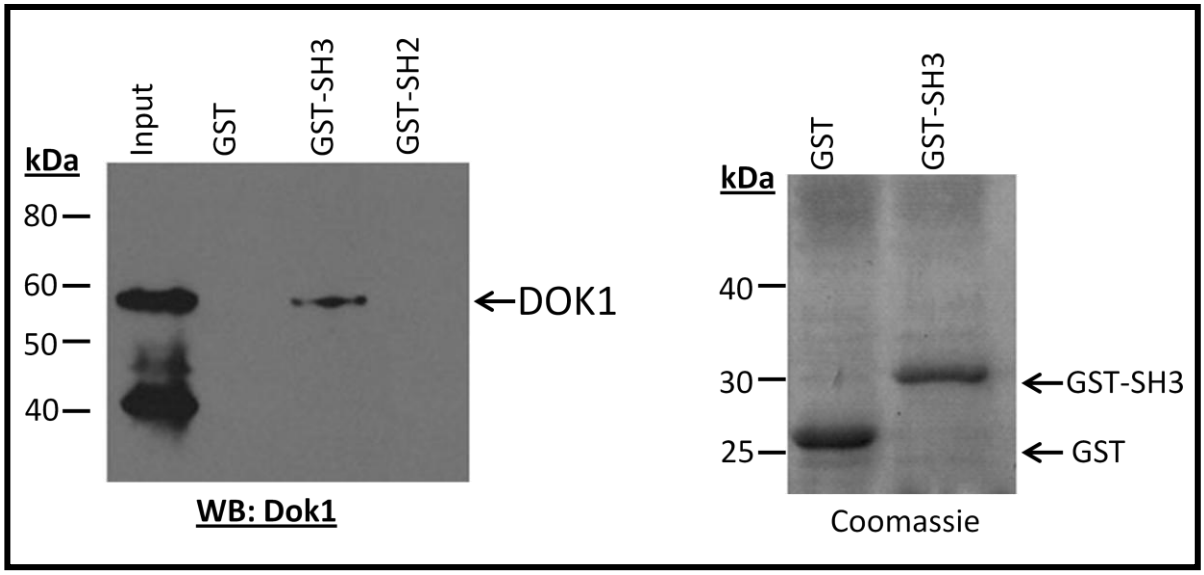

FIGURE 4.22: Dok1 associates with SRMS SH3 domain. A GST-Pull-down assay was conducted with the GST-SH3 and GST-SH2 domains of SRMS using HEK293 cell lysates. Bound proteins were resolved via SDS-PAGE and immublotted with anti-Dok1 antibodies (left panel). Expression of the bacterially expressed proteins is shown via Coomassie staining of the PAGE gel (right panel). Similar results were obtained in at least three different experiments.

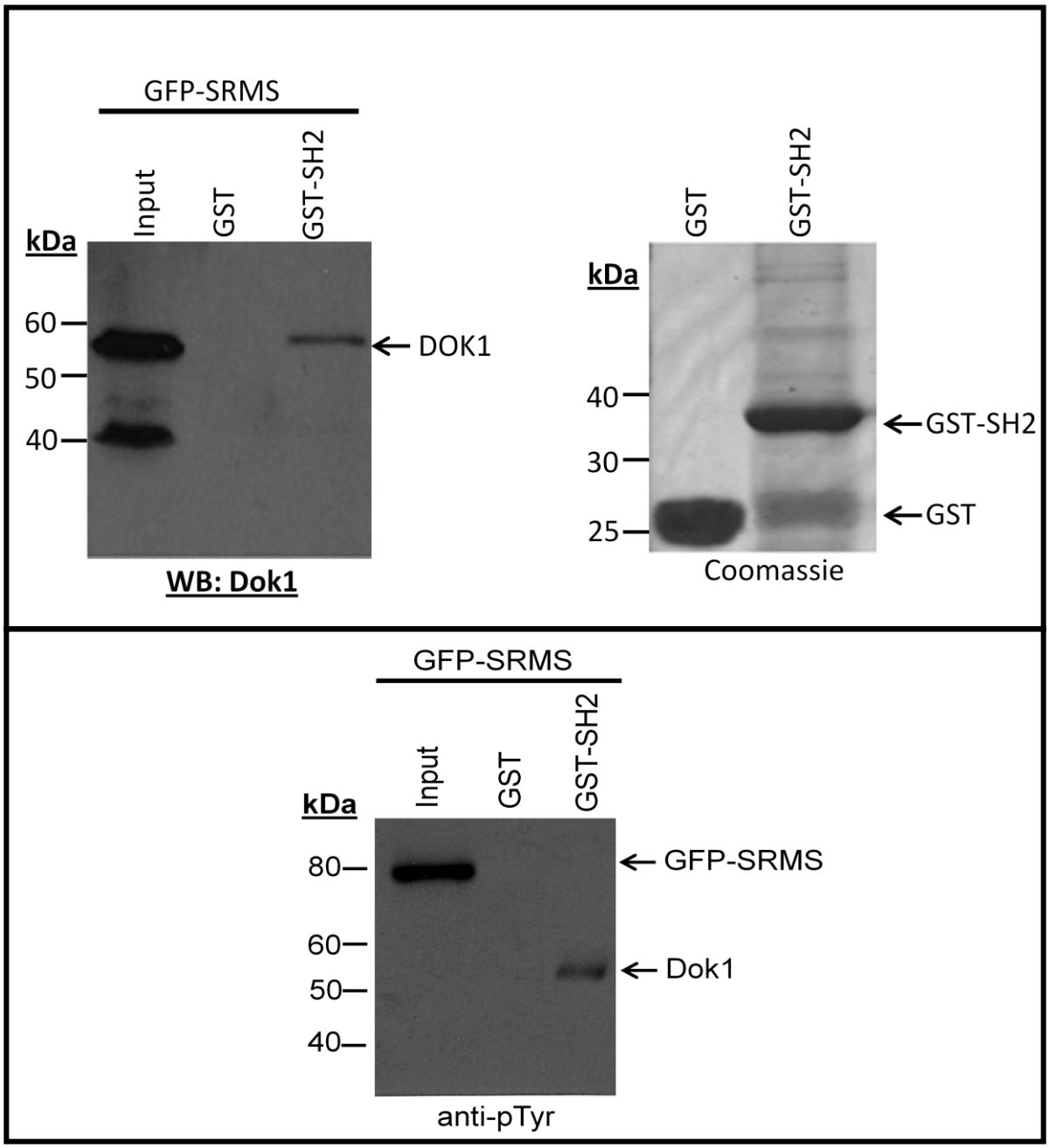

FIGURE 4.23: Tyrosine phosphorylated Dok1 associates with SRMS SH2 domain. HEK293 cells transfected with GFP-SRMS, were lysed and utilized towards a GST-pull down assay with the SRMS GST-SH2 domain. Bound proteins were resolved via SDS-PAGE and immunoblotted with anti-Dok1 antibodies (top left panel) and phosphotyrosine antibodies (bottom panel). Expression of the GST-fused proteins is shown in the Coomassie stained image of the SDS-PAGE gel (top right panel). Similar results were obtained in at least three different experiments.

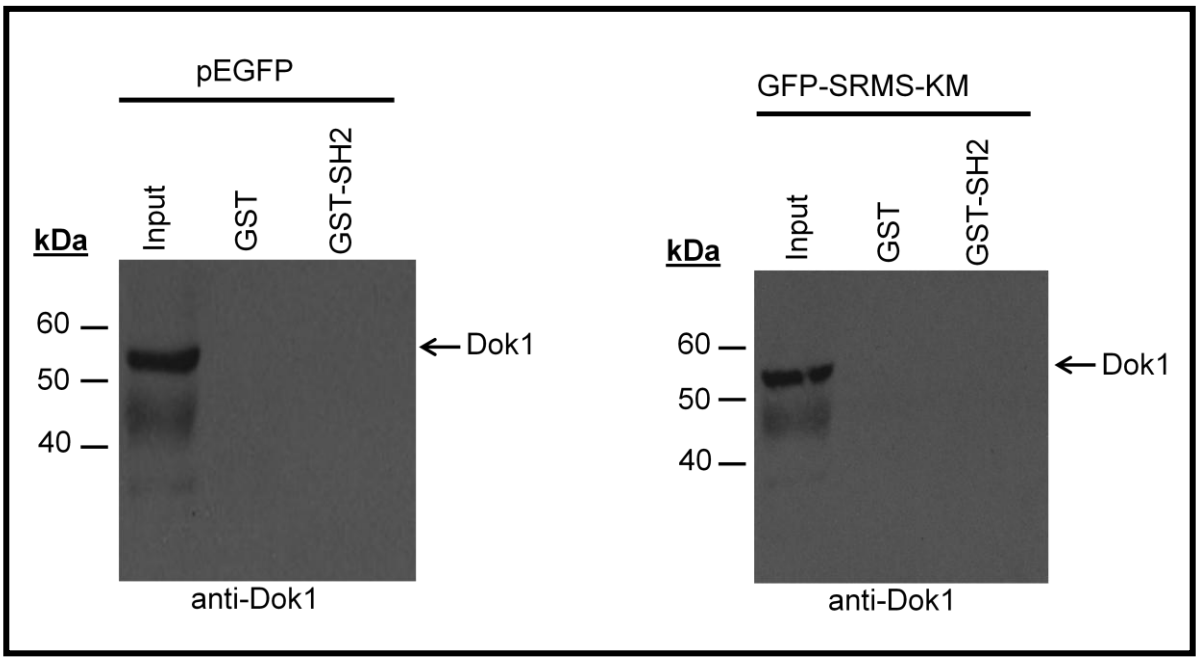

FIGURE 4.24: Tyrosine phosphorylation is necessary for the association of Dok1 with SRMS SH2 domain. A GST-pull down assay was performed using lysates derived from HEK293 cells transfected with either pEGFP vector or GFP-SRMS-K258M. Bound proteins were resolved via SDS-PAGE and immublotted with anti-Dok1 antibodies Similar results were obtained in at least three different experiments.

4.4.2 Validation of Dok1 as a substrate of SRMS

Dok1 was identified in a high-throughput proteomics screen as a potential substrate of Src-family kinases as well as SRMS. In order to begin to characterize the substrate-specificity of Dok1 towards SRMS, HEK293 cells were transfected with GFP-Dok1 alone or co-transfected with GFP-Dok1 and GFP-SRMS. Dok1 was then immunoprecipitated from the lysates and subjected to immunoblotting against phosphotyrosine, Dok1 and SRMS antibodies. Immunoblotting with anti-phosphotyrosine antibodies revealed tyrosine phosphorylation of Dok1 in the presence of SRMS (Fig 4.25, lane 4, top panel**).** Immunoblotting with anti-SRMS antibodies revealed the co-precipitation of SRMS with Dok1, suggestive of binding associations between both the proteins (Fig 4.25, lane 4, middle panel**)**. These results, therefore, confirmed the phosphorylation of Dok1 in the presence of SRMS and were also suggestive of potential binding interactions between both proteins.

To determine the phosphorylation of endogenous Dok1 by SRMS, GFP-tagged wild-type SRMS or the catalytically-inactive, ΔN-SRMS, were transiently expressed in HEK293 cells and endogenous Dok1 was immunoprecipitated to assess for tyrosine phosphorylation. The aim of such an experiment was to determine whether ectopically expressed SRMS induces the phosphorylation of endogenous Dok1 and also whether the N-terminal region of SRMS regulates the mechanism of its substrate recognition. Immunoblotting via anti-phosphotyrosine antibodies revealed endogenous Dok1 to be phosphorylated upon ectopic expression of SRMS (Fig 4.26, lane 4, bottom panel). Furthermore, immunoblotting with an antibody against GFP revealed that, like wild-type SRMS, ΔN-SRMS also co-immunoprecipitated with endogenous Dok1, thereby attesting data from Figure 4.21 that the N-terminus is expendable in directing substrate recognition and association (Fig 4.26, lane 1, middle panel**)**.

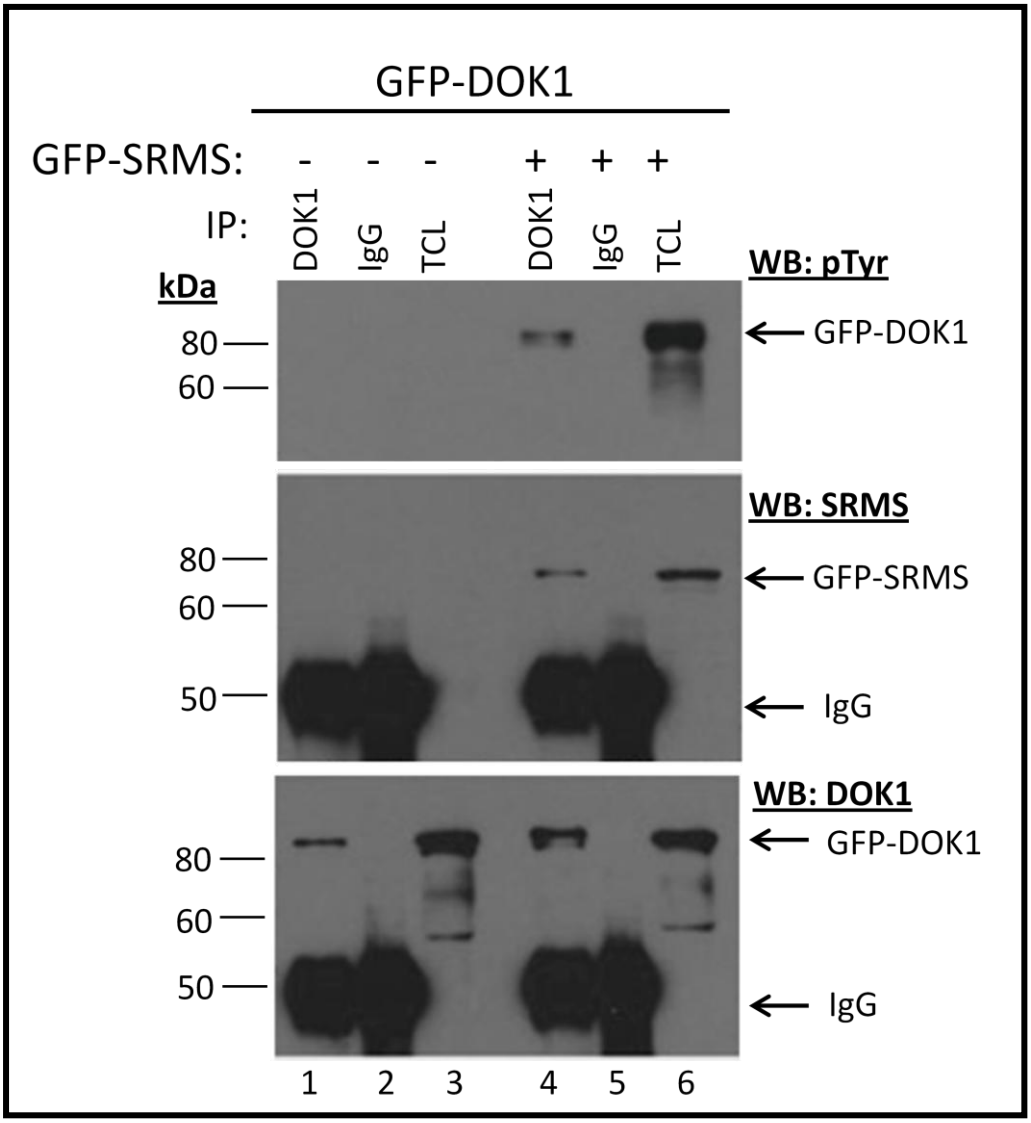

FIGURE 4.25: SRMS induces the phosphorylation of Dok1 in cells. Dok1 was immunoprecipitated from GFP-Dok1 alone or GFP-Dok1/GFP-SRMS co-transfected cohorts of HEK293 cell lysates and probed for tyrosine phosphorylation using anti-phosphotyrosine antibodies (top panel), SRMS (middle panel) and Dok1 (bottom panel). Anti-IgG (rabbit) was used as control. Total cell lysates were used to indicate the relative expression of both proteins. Similar results were obtained in at least three different experiments.

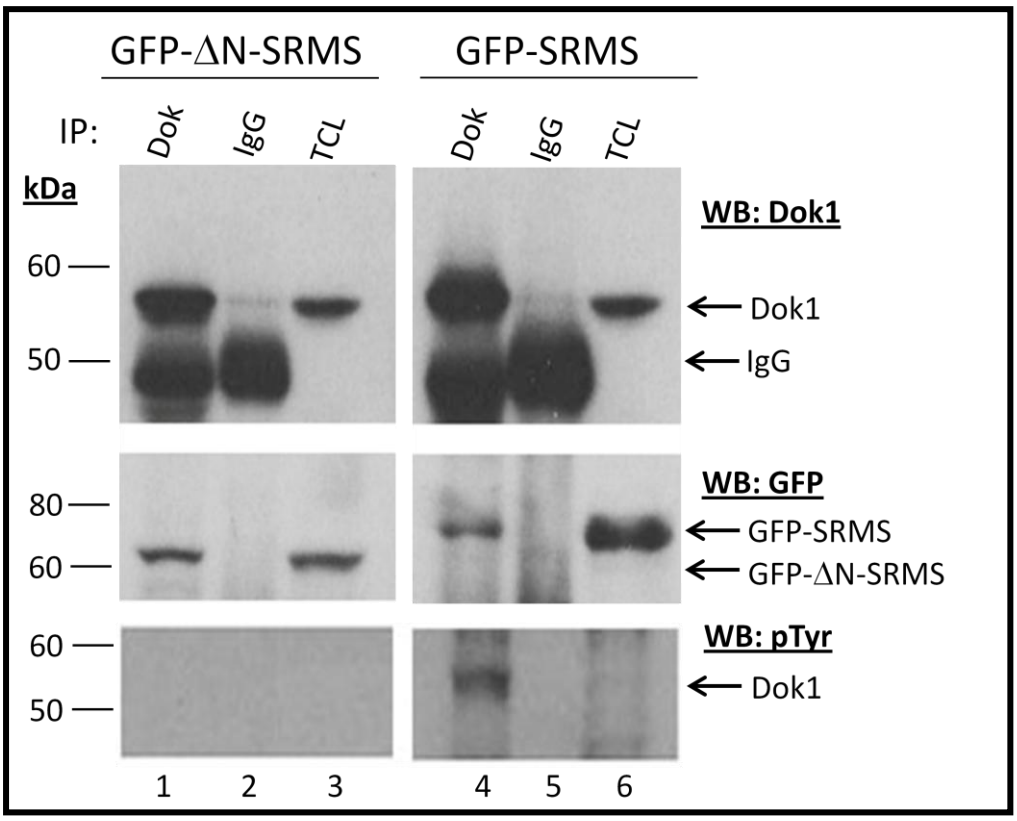

FIGURE 4.26: Ectopically expressed SRMS induces the phosphorylation of endogenous Dok1. Dok1 was immunoprecipitated from GFP-SRMS or GFP-ΔN-SRMS transfected cohorts of HEK293 cell lysates and immunoblotted for Dok1 (top panel), SRMS (middle panel) and phosphotyrosine (bottom panel). Total cell lysates were used to indicate the relative expression of the respective proteins. Anti-IgG (rabbit) was used as control. Similar results were obtained in at least three different experiments.

4.4.2.1 Mapping the regions on Dok1 phosphorylated by SRMS

Dok1 possesses a dense array of prolines and tyrosine residues along its C-terminal segment that extends from the phospho-tyrosine binding (PTB) domain into a 222 amino acid chain. While several proline residues lie clustered herewith, these remain interspersed between a total of 10 tyrosine residues. In order to map the cluster of tyrosine residues phosphorylated by SRMS, a total of five C-terminally truncated mutants of Dok1 were generated, each successively containing an increasing number of tyrosine residues (Fig 4.27).

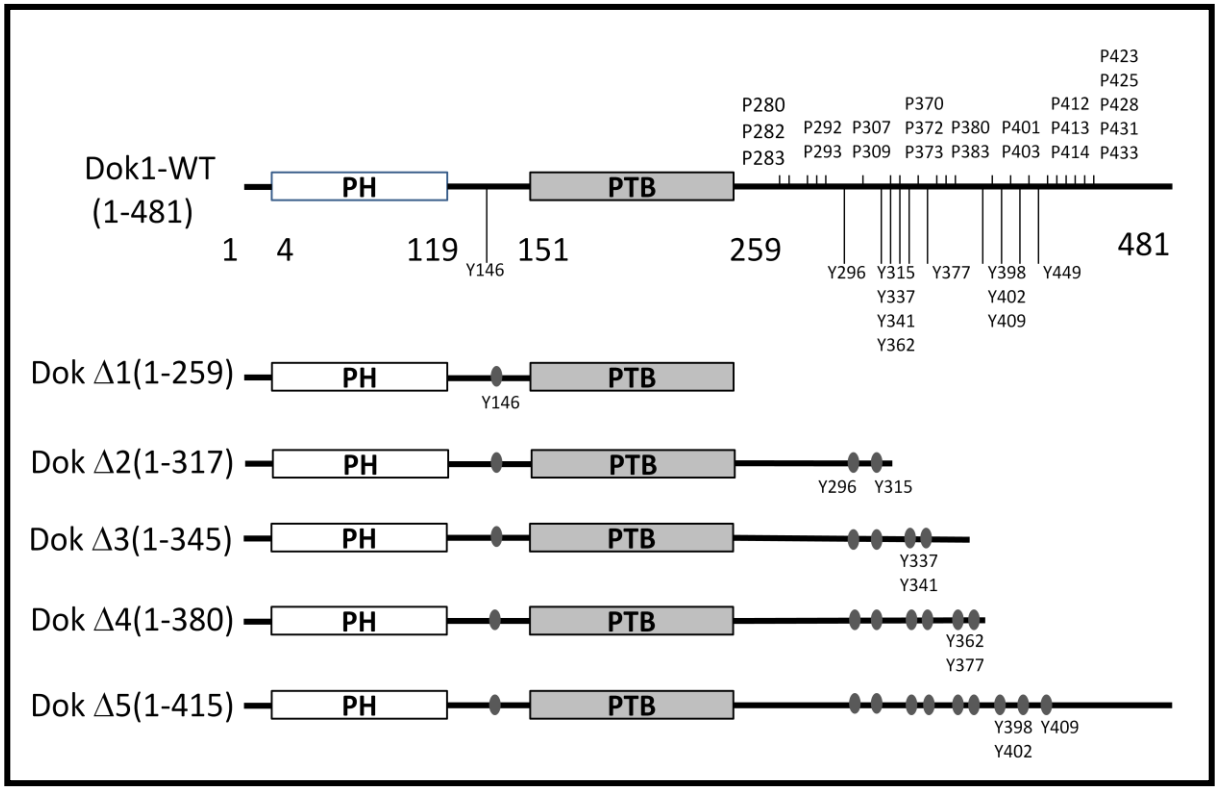

FIGURE 4.27: Mapping Dok1 Tyrosine residue clusters. A Schematic diagram of wild type Dok1 (Dok1 WT) and its five, C-terminally truncated, mutants showing the progressively increasing number of tyrosine and proline residues with each mutant.

Wild-type Dok1 as well as its mutants were transiently transfected in HEK293 cells, either alone or with mCherry-SRMS. The Dok1 mutants were immunoprecipitated with anti-GFP antibodies and the immunoprecipitates and total cell lysates were immunoblotted with antibodies against phosphotyrosine, GFP and SRMS. Immunoblotting the total cell lysates with anti-phosphotyrosine antibodies revealed that, besides wild-type Dok1 (Fig. 4.28, lane 12), Dok-Δ3 (1-345), Dok-Δ4 (1-380) and Dok-Δ5 (1-415) were all phosphorylated in the presence of mCherry-SRMS (Fig. 4.28, middle panel). Barring Dok-Δ1(1-259)· as the only exception, Dok-Δ2 (1-317) was also found to be phosphorylated upon longer exposure of the X-ray film to the immunoblotted membrane. The expression levels of transfected GFP-Dok1 mutants and mCherry-SRMS proteins are shown in Figure 4.28 (top and bottom panels, respectively). Immunoblotting performed on the immunoprecipitates also demonstrated identical results (Fig. 4.29, top panel) which confirmed that all Dok1 mutants, except for Dok-Δ1, were phosphorylated in the presence of ectopically expressed mCherry-SRMS. Furthermore, that co-immunoprecipitation of ectopic SRMS progressively diminished with the smaller Dok1 mutants (Fig 4.29, bottom panel, lanes 1-6), potentially indicates the underlying significance of the C-terminal proline and phosphotyrosine residues in mediating binding interactions with SRMS.

It was vital to also determine whether Dok1 is a direct substrate of SRMS since Dok1 is essentially a scaffolding protein that provides for a docking platform to mediate interactions between several proteins. Therefore, the hypothesis that Dok1 phosphorylation could be mediated by SRMS via activation of a third protein seemed plausible. An *in vitro* kinase assay, using the purified recombinant kinase (GST-SRMS) and substrate (GST-Dok1), in the presence or absence of ATP, revealed that Dok1 is a direct bonafide substrate of SRMS (Fig. 4.30). GST alone was not phosphorylated in the presence of SRMS kinase (Fig. 4.31).

Overall, through the employed experimental procedures involving site-directed mutagenesis, immunoprecipitation, immunofluorescence microscopy/ immunocytochemistry, *in vitro* GST-pull-down assays and kinase-substrate assays, this thesis concludes with the characterization of the catalytic activity of SRMS as well as the characterization of Dok1 as its first substrate.

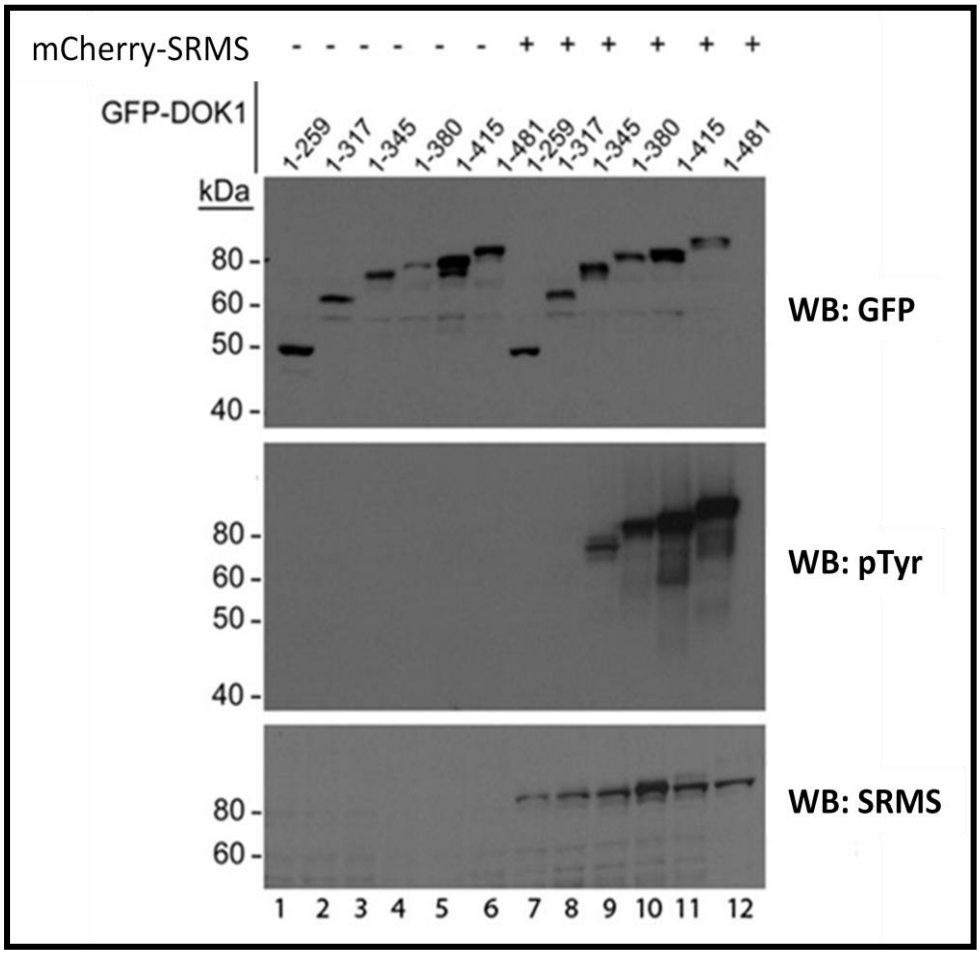

FIGURE 4.28: Tyrosine phosphorylation of Dok1 mutants in the presence of SRMS. All five Dok1 mutants along with wild type Dok1 were transfected either alone or co-transfected with mCherry-SRMS in HEK293 cells and total cell lysates were used for immunoblotting with antibodies against GFP (top panel), phosphotyrosine (middle panel) and SRMS (bottom panel). Similar results were obtained in at least three different experiments.

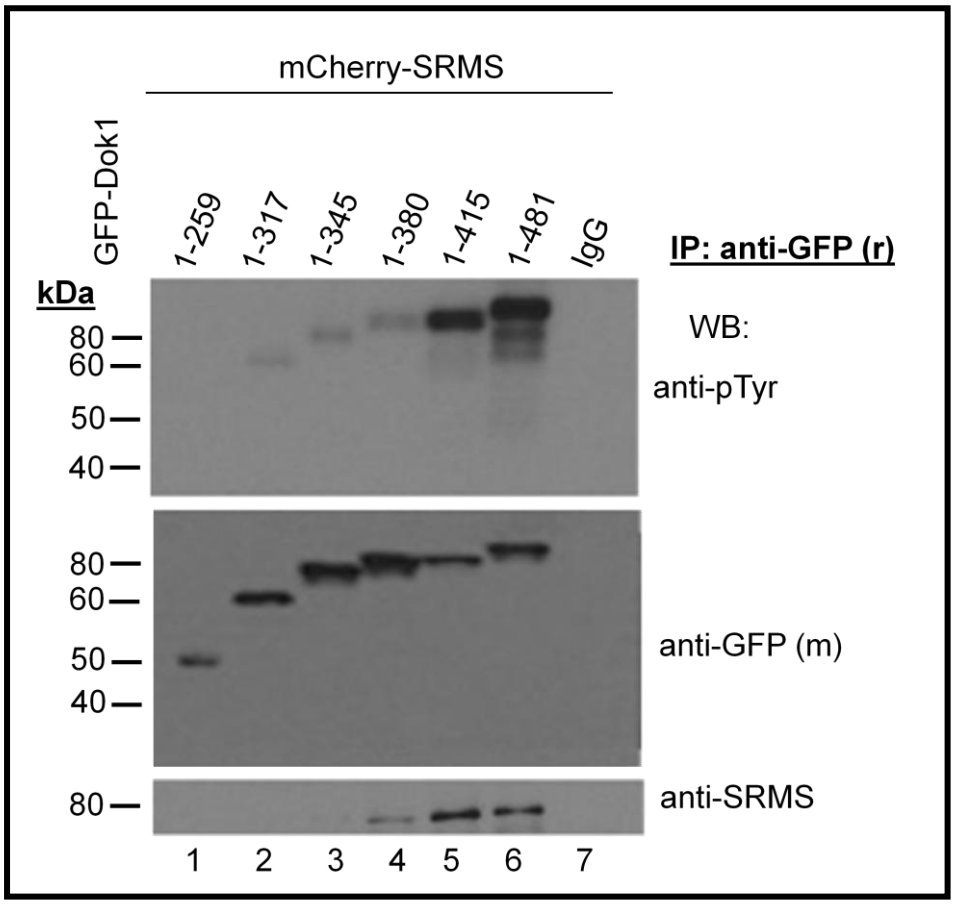

FIGURE 4.29: Immunoprecipitation analysis depicting the tyrosine phosphorylation of Dok1 mutants in the presence of SRMS. HEK293 cells were co-transfected with GFP-tagged wild type Dok1 or its five mutants and mCherry-SRMS and subjected to immunoprecipitation with anti-GFP (rabbit) antibodies. Immunoprecipitates were probed for tyrosine phosphorylation using antibodies against phosphotyrosine (top panel), for Dok1 using anti-GFP (mouse) (middle panel) and for SRMS using anti-SRMS (bottom panel). Similar results were obtained in at least three different experiments.

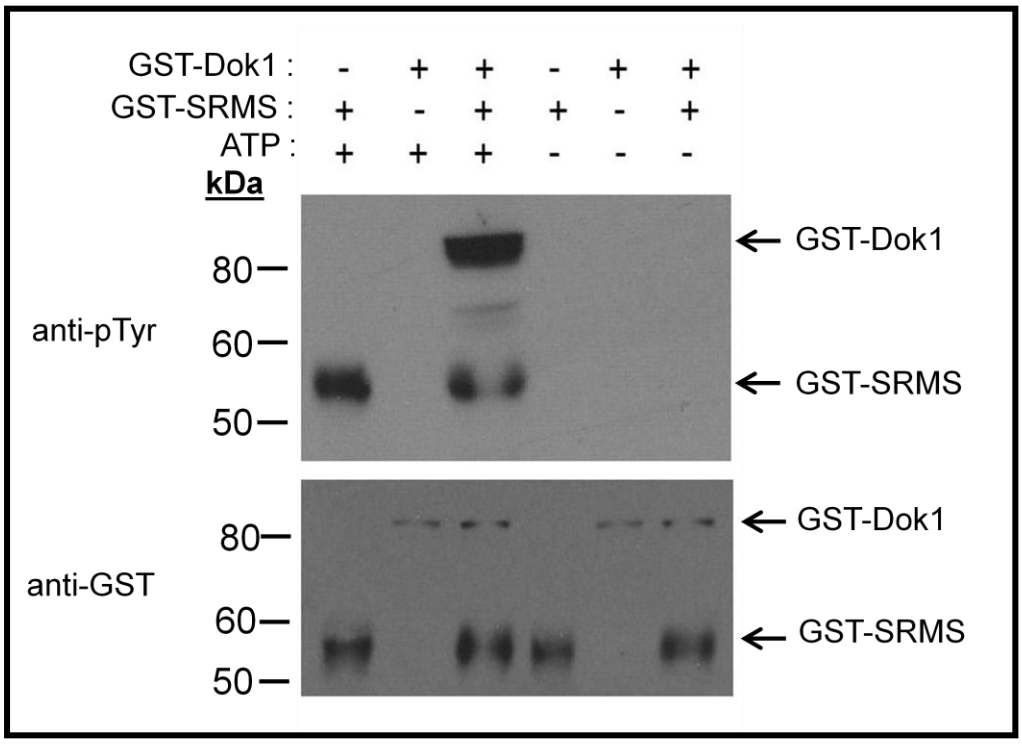

FIGURE 4.30: Dok1 is a direct substrate of SRMS. An *in vitro* kinase assay was performed using the active kinase domain, GST-SRMS, and the substrate, GST-Dok1, in the presence or absence of ATP. Tyrosine phosphorylation was probed via anti phosphotyrosine antibodies (top panel) and expression of the GST-fused proteins, via anti-GST (bottom panel). Similar results were obtained in at least three different experiments.

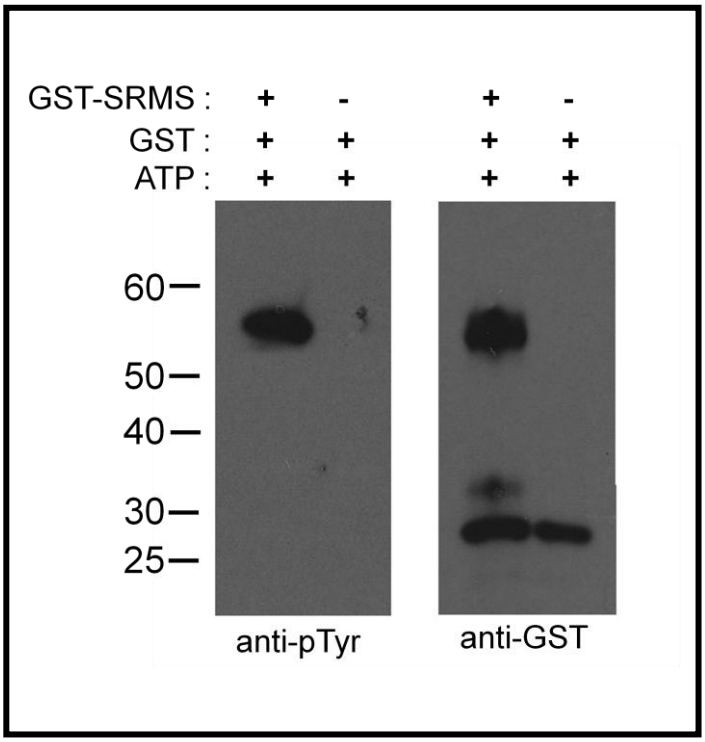

FIGURE 4.31 SRMS kinase does not phosphorylate GST alone. A control *in vitro* kinase assay was performed using the active kinase, GST-SRMS and GST alone in the presence of ATP. Tyrosine phosphorylation was probed via anti phosphotyrosine antibodies (left panel) and expression of the GST or GST-SRMS, via anti-GST (right panel). Similar results were obtained from two different experiments.

5. DISCUSSION

The non-receptor tyrosine kinase SRMS was cloned in 1994, but the autoregulatory mechanisms that dictate its enzymatic activity, substrate identification and recognition have never been examined. As well, the expression profile of SRMS in cells derived from the normal or cancer tissues has never been determined. The present study is the first to investigate and unravel that (a) SRMS is potentially overexpressed in the majority of breast cancer cell lines studied, (b) that its catalytic activity is primarily regulated by the N-terminal region, completely divergent from BRK and FRK, and that (c) the adapter protein Dok1 is its first novel physiological substrate.

5.1 SRMS and Dok1 expression and intra cellular localization in breast cancer cell lines.

The expression of BRK and FRK has been previously reported. Such analyses reveal that while BRK is overexpressed in over 70% of breast carcinomas, FRK is potentially underexpressed in breast cancers (Barker *et al.*, 1997; Cance *et al.*, 1994; Craven *et al.*, 1995). However, to date, the expression pattern of SRMS remains uncharacterized in breast cancers. This study presents evidence for SRMS as a potentially over-expressed protein, akin to BRK, in different breast cancer cell lines. The study covered a relatively broad spectrum of cell lines derived from the human breast cancer tissues to survey the expression of SRMS. Data from Fig 4.1 show that SRMS expression is elevated in the breast cancer cell lines evaluated, along the lines of BRK, but reduced in the normal breast cell line, 184B5. This preliminary study, thus, suggests that SRMS is potentially upregulated in breast cancers as compared to normal mammary tissues which is reflective of its potential involvement in the molecular mechanisms associated human breast cancer. It was also observed that the magnitude of SRMS expression did not vary significantly in the breast carcinomas as opposed to that of BRK, which for the latter, is consistent with previous reports (Barker *et al.*, 1997).

While SRMS expression, like BRK, was generally elevated in the breast cancer cell lines, a reduced expression of the protein in the non-tumorigenic/ normal breast epithelial cell line, 184B5, was observed. Established from a reduction mammoplasty, and chemically immortalized via Benzo(a)pyrene, the 184B5 cell line is a non-malignant human mammary epithelial cell line which exhibits no symptoms of tumorigenesis/malignancy. With the exception of AU565 and MDA-MB-468 cell lines, SRMS exhibited a generally elevated expression pattern in the breast cancer repertoire examined in this study. However, SRMS

expression appeared elevated in the Estrogen Receptor (ER) positive HBL100 cell line. The expression profile of SRMS, thus, seems similar to BRK in that the majority of carcinoma cell lines exhibit high levels of the protein. Such an observation may invite knowledge on the genetic loci of the corresponding genes. SRMS resides in the chromosomal locus 20q13.33, which is 1.5 kb upstream of the BRK gene. Such an arrangement of the genes is reflective of a potentially tight genetic linkage between BRK and SRMS. Furthermore and more importantly, their genetic loci also correspond to a region that is frequently amplified in breast cancer (Harvey and Crompton, 2004) indicating that like BRK, expression of SRMS may also potentially be abberrantly upregulated in breast carcinomas.

However, unlike BRK, the present data on the expression of SRMS in a limited panel of breast cancer cell lines currently impedes any attempts to establish a correlation between the expression of the PTK and either the cancer molecular subtype or even an explicit morphological characteristic pertaining to the cell growth and proliferation. This indicates a need to investigate the expression of SRMS at the transcriptional and post-transcriptional level in a larger panel of breast cancer cell lines and tissues with varying clinical/pathological facets. Immunohistological analyses on a broad spectrum of breast tissue specimens should serve to ascertain the expression of SRMS in breast carcinomas and its molecular association with the pathological grade of the specimens and the overall etiology of the disease.

We have also presented evidence of Dok1 expression in a subset of breast cancer cell lines. Although Dok1 has been examined for mutations that affect its expression in other cancer types (Mercier *et al.*, 2011; Saulnier *et al.*, 2012; Siouda *et al.*, 2012), to our knowledge its expression has not been reported. We identified a differential pattern of Dok1 expression in the eight breast cancer cell lines, amongst which, akin to SRMS, an elevated expression was witnessed in the HBL-100 cell line (Fig 4.15). Notably though, in the AU565, MDA-MB-468 and SK-BR3 cell lines, Dok1 was either too low or absent. Considering that Dok1 is a candidate tumor suppressor, its expression profile might be clinically significant if investigated on a wider panel of breast cancer cell lines.

The sub-cellular localization of endogenous SRMS in the breast cancer cell lines studied was determined to be predominantly punctate and cytoplasmic in nature (Fig 4.2). Besides the breast cancer cell lines, MDA-MB-231 and AU565, a similar punctate and cytoplasmic localization was also observed in the cervical cancer cell line, HeLa. However, it is possible that this intracellular distribution pattern may vary in the other breast cancer cell lines and

that such deviation in the sub-cellular distribution might be potentially associated with discrete functionalities specific to certain cell lines. This may further be dependent on SRMS interactions with specific endogenous proteins, organelles or membranes via its functional SH3 and/or SH2 domains. Even as the endogenous levels of SRMS appears low in AU565 (Fig 4.1), immunocytochemistry analysis yields a strong fluorescent signal for SRMS in the same cell line. This is probably because immunocytochemistry is not necessarily a quantitative. Our fluorescence microscopy results in Fig. 4.12 - 4.14 show that the localization of SRMS may be dictated by interactions involving the extended N-terminus or the SH2 domain. The deletion of any of these regions at least partially altered the cytoplasmic localization of SRMS from punctate to diffuse. It is possible that the N-terminal region and the SH2 domain mediate intermolecular interactions with other target proteins or molecules (e.g. lipids) that sequester SRMS to distinct punctate structures within the cell. However, how these critical regions mediate such associations with other cellular components is as yet unknown. Intermolecular interactions involving the SH2 and SH3 domains of Src family kinases are also known to regulate the sub-cellular localization of these kinases (Mayer *et al.*, 1995; Qiu and Miller, 2004; Thomas and Brugge, 1997).

The localization of the inactivating K258M SRMS mutant was cytoplasmic and diffused in all cells. It is not obvious why mutating the ATP binding site would alter the intracellular localization of SRMS. This observation therefore requires further investigation. The cellular localization of BRK, for example, may not be as tightly regulated. The protein has been shown to associate with its nuclear substrate, Sam68, in certain breast carcinomas besides the normal human prostate epithelial cells and well-differentiated prostate carcinomas, but localizes to the cytoplasm in poorly differentiated prostate tumors (Brauer and Tyner, 2010). As well, strategically targeting BRK specifically to the membrane has been shown to enhance its oncogenic abilities, a feature that functionally contrasts with its nuclear-targeted counterpart (Ie Kim and Lee, 2009). Nonetheless, it is yet to be determined how the localization of SRMS may affect its function.

Although Dok1 predominantly localizes to the cytoplasm/membrane, a few published reports have indicated a nuclear distribution of the protein as well (Lee et al., 2004b; Noguchi et al., 1999b). Our data in figure 4.17 and 4.18 indicates that the intracellular localization of Dok1 is predominantly cytosolic as analyzed in the breast cancer cell lines, MDA-MB-231 and MDA-MB-435 as well as in the cervical cancer cell line, HeLa. Dok1 possesses a nuclear export signal (NES) that resides within a cluster of amino acids

348-359 situated at the C-terminal tail. Published reports suggest that Dok1 shuttles between the nucleus and the cytoplasm, and such intracellular mobility is regulated via phosphorylation of Dok1 (Niu *et al.*, 2006). Along these lines, Woodring *et al.* reported that Dok1 phosphorylation by c-Abl tyrosine kinase promotes the localization of Dok1 to the filopodia tips in actin microstructures of mouse embryonic fibroblasts to allow for enhanced cell spreading (Woodring *et al.*, 2004a). Likewise, Dok1 was also shown to localize predominantly to the membrane via its PH domain following phosphorylation by v-Src as well as stimulation with insulin (Noguchi et al., 1999b). It is possible that the cytoplasmic/membrane localization of Dok1 in breast cancer cell lines might promote similar functions. Nonetheless, this hypothesis is yet to be investigated.

5.2 Regulation of SRMS tyrosine kinase activity

Through site-directed mutagenesis and conventional cloning strategies, we have delineated the innate regulatory features that govern the catalytic activity of SRMS. We determined that unlike the other Src-related tyrosine kinases, the catalytic activity of SRMS is regulated via its N-terminal region. We report that deleting the entire 51 amino acid spanning N-terminal region abrogates the tyrosine kinase activity of SRMS. This specifically contrasts the regulatory mechanism proposed for the c-Abl tyrosine kinase whose autoinhibitory intramolecular interactions are relieved upon terminating the 81 amino acid N-terminus region, thereby activating the enzyme (Nagar *et al.*, 2003; Pluk *et al.*, 2002). Furthermore, this also distinguishes SRMS from its other 2 family members, BRK and FRK wherein the modes of catalytic auto-regulation, unlike the N-terminus, depends instead on the inhibitory C-terminal tail possessing a regulatory tyrosine residue.

The catalytic activity of Src family kinases is regulated by intramolecular interactions. Studies on BRK have also shown that like Src kinases, BRK is regulated negatively by phosphorylation of C-terminal tyrosine 447 (which is analogous to the regulatory Y530 of human Src) and positively by phosphorylation of tyrosine 342 in the catalytic domain (as with Y419 of human Src) (Derry *et al.*, 2000a; Qiu and Miller, 2002). c-Src tyrosine kinase (Csk) regulates Src family kinase activity by phosphorylating their C-terminal tyrosines thereby promoting intramolecular interactions that inactivate these enzymes (Okada and Nakagawa, 1989). Mutating the C-terminal tyrosine to phenylalanine in BRK (BRK-Y447F) and Src family kinases results in the constitutive activation of the enzymes. SRMS lacks a C-terminal regulatory tail and our data is the first to report that full-

length SRMS is active, as revealed by autophosphorylation and phosphotyrosine content as a measure of protein kinase activity in cells (Fig 4.7 and 4.8). However, the full-length SRMS induced a relatively lower level of phosphorylation of cellular targets compared with BRK-Y447F (Fig. 4.7). Speculations to this end may be drawn from the observation that SRMS is sequestered in specific cytoplasmic compartments within the cell and has lower access to cellular substrates compared with BRK which may freely shuttle between the nucleus and cytoplasm.

We generated a series of SRMS mutants to understand the autoregulatory mechanism of the kinase. Deletion of the entire 51 amino acid spanning N-terminus region completely abrogated catalytic activity. BRK has a much shorter N-terminus and unlike SRMS, the N-terminal amino acid sequence preceding the SH3 domain in BRK is known to be dispensable for the regulation of kinase activity (Qiu and Miller, 2004). As well, contrasting with SRMS, the deletion of the 81 amino acid N-terminus region of c-Abl relieved the enzyme of its autoinhibitory intramolecular interactions, thereby activating the enzyme (Nagar *et al.*, 2003; Pluk *et al.*, 2002). Interestingly, deletion of the SH2 domain of SRMS also drastically reduced kinase activity (Fig. 4.12). On the contrary, Qiu and Miller found that the Y447F and ΔSH2 variants of BRK induced comparable and higher levels of substrate phosphorylation, which implied that wild type BRK is potentially maintained in an inactive (auto-inhibited) conformation by the putative SH2-pY447 interaction. While the BRK-SH2 and SH3 domains play an inhibitory role, our data shows that the SH2 domain in SRMS is essential for the enzyme to sustain its catalytic activity in a yet unidentified mechanism. Thus the autoregulatory mechanism of SRMS is distinct from that of BRK and Src kinases wherein the modes of catalytic autoregulation depends on the inhibitory C-terminal tail possessing a regulatory tyrosine residue (Brauer and Tyner, 2009, 2010).

W260 and W184 situated within the SH2-kinase linker in Src family kinases and BRK respectively, have been shown to play opposing roles in maintaining the interactions of the SH2 and SH3 domains with their respective intramolecular ligands (LaFevre-Bernt *et al.*, 1998; Qiu and Miller, 2004). The crystal structure of inactive Hck revealed that W260 interacts with its kinase domain, maintaining the kinase in an inactive conformation (Sicheri *et al.*, 1997). Thus, the W260A mutation in Hck activated the enzyme (LaFevre-Bernt *et al.*, 1998). However, the analogous W184A mutation in BRK was found to abrogate the kinase activity of the enzyme (Qiu and Miller, 2004). The effect of W184A is reminiscent of the analogous W342A mutation in c-Raf, which also resulted in the loss of kinase activity

(McPherson *et al.*, 2000). We found that the analogous W223A mutation in SRMS also caused a loss of kinase activity (Fig. 4.7 and 4.8). Thus, similar to BRK, W223 in SRMS is predicted to interact with the kinase domain and promote the active conformation of the enzyme.

We also found that mutating the three proline residues within the SH2-kinase linker region in SRMS, did not significantly affect its kinase activity. This contrasts with BRK as well as Src family kinases, wherein mutating the linker region proline residues resulted in significantly higher tyrosine kinase activity owing to the destabilization of autoinhibitory intramolecular interactions (Briggs and Smithgall, 1999; Qiu and Miller, 2004; Sicheri *et al.*, 1997). These proline residues in SRMS are not conserved with either BRK or the Src family kinases. Since mutating these proline residues did not affect the tyrosine kinase activity of SRMS, it indicates an indispensable role for these residues in intramolecular binding.

Furthermore, while our strategy to specifically delete a 13 amino acid proline-rich segment in the N-terminal region of SRMS (PΔN SRMS) failed to alter the kinase activity of SRMS, we propose to investigate the involvement of the 38 amino acid sequence that precedes this proline-rich segment. We shall therefore investigate other SRMS mutants to be generated along these lines.

5.3 Phosphorylation of Dok1 by SRMS

We have also identified the first direct substrate of SRMS, Dok1, an adapter protein that is phosphorylated downstream of several tyrosine kinases (Hence the name, **D**ownstream **o**f tyrosine **k**inase1). Previous studies with Src have revealed that Src phosphorylates Dok1 on Y362, Y398 and Y449 (Niu *et al.*, 2006). Other studies have also indicated Dok1 as a constitutively tyrosine phosphorylated target of Bcr-Abl in chronic myelogenous leukemia (CML) progenitor cells (Carpino *et al.*, 1997). Our data indicates that Dok1 is phosphorylated on several tyrosine residues along its C-terminal tail (Fig. 4.31 and 4.32). We observe tyrosine phosphorylation signals for the Dok1 mutants, especially DokΔ4, and DokΔ5, in a manner that is reflective of the number of tyrosine residues that each mutant possesses. Also, our data indicates that the first Dok1 mutant (DokΔ1), comprising a single tyrosine residing between the PH and the PTB domains, is not detectably phosphorylated by SRMS. It may be imperative to note that this mutant lacks the entire C-terminal tail and thus

the proline-rich motifs which may be essential for binding interactions with SRMS. In view of this, binding interactions with SRMS were visibly diminished even for DokΔ2 and DokΔ3, indicating that the prolines in the C-terminal tail of Dok1 are crucially needed for associating with SRMS. This may also, in part, explain the reduced levels of tyrosine phosphorylation of these two Dok1 mutants.

Still, the strategy seemed ineffective in specifically identifying the residues that are phosphorylated by SRMS since all four C-terminal proline-containing mutants display tyrosine phosphorylation. This is an obvious caveat of the strategy which we propose can be resolved via site-directed mutagenesis, generating a series of constructs with successive mutations in all the 11 tyrosines.

6. FUTURE DIRECTIONS

6.1 Expression of SRMS in breast cancer cell lines and tissues

From the results presented in Fig 4.1, SRMS expression appeared to be elevated in breast cancer cells compared to cells derived from the normal mammary tissues. It will be important to determine the expression of SRMS in a larger panel of cell lines derived from both, normal and cancererous breast tissues to expand upon the findings reported by this study. Immunohistochemistry analyses pursued on a wide panel of tissue microarrays comprising breast cancer and matched normal tissue sections, shall further aid in characterizing the physiological significance of SRMS expression in breast cancer etiology. Such efforts shall particularly help correlate and validate SRMS expression with perhaps breast cancer molecular subtypes and/or the pathological grade, characterized by the invasiveness and tumorigenecity of the disease.

6.2 Characterization of SRMS cytoplasmic puncta

Based on the analyses of endogenous SRMS distribution in the breast cancer cell lines studied thus far, a predominantly punctate cytoplasmic pattern was observed (Fig 4.3 and 4.5). A similar pattern was also observed with ectopically expressed SRMS as studied in HEK293 cells (Fig 4.4). It is possible that the N-terminal sequence, the SH3 and/or the SH2 domains of SRMS, via intermolecular interactions with endogenous targets, may sequester it to specific cellular compartments. Furthermore, SRMS may either directly or indirectly associate with specific cellular organelles or vesicles such as the endoplasmic reticuli, mitochondria, lysosomes/endosomes. For instance, similar cytoplasmic puncta formation has been previously reported for FRK that is believed to associate with EGFR in cellular endosomes (Jin and Craven, 2013). Cytoplasmic punctate structures are also known to form in Anaplastic lymphoma kinase (ALK)-transformed cells where its association with characteristic cytoplasmic foci, comprising mRNA and mRNA-processing proteins, has been described (Fawal *et al.*, 2011). Furthermore, the association of microtubule-associated protein 1 light chain 3 (abbreviated LC3) with autophagosomal membranes also yield cytoplasmic puncta during the induction of autophagy (Mizushima *et al.*, 2010). Thus, there exist several incentives for exploring the punctate distribution exhibited by SRMS. Commercially available molecular tracker/probes exhibiting immunospecificity towards

certain cellular organelles, such as those described above, may be procured and utilized to aid the characterization of SRMS puncta.

6.3 Three-dimensional structural analysis of SRMS and tyrosine phosphorylation analyses via mass spectrometry

To date, there have been no reports describing the three-dimensional structure of either full-length SRMS or its functional domains. Structural analysis of c-Src has revealed significant information on the intramolecular interactions of the kinase involving its functional domains including the auto-regulatory regions(Cowan-Jacob *et al.*, 2005). For an enzyme, such information presents vital clues about its intrinsic functional characteristics pertaining to auto-catalytic regulation, substrate recognition/binding and other diverse facets of intracellular behaviour which may be crucial to understand the cellular roles of the enzyme.

Recent investigations on c-Abl tyrosine kinase have revealed an autoinhibitory mechanism wherein the N-terminal cap preceding the SH3 domain interacts with the SH2 kinase linker region to inhibit the catalytic activity of the kinase (Corbi-Verge *et al.*, 2013; de Oliveira *et al.*, 2013). Our data demonstrates that unlike BRK and Src family kinases, SRMS catalytic activity is apparently positively regulated via its N-terminal region and the SH2 domain. However, like BRK, SRMS catalytic activity is attenuated in its W223A mutant. It has been speculated that this tryptophan residue, conserved across Src and FRK/PTK6 family kinases, interacts with the kinase domain in BRK thereby stabilizing the active conformation of the PTK (Qiu and Miller, 2004). Furthermore, the functional domains of SRMS, namely the SH3, SH2 and the kinase domain, exhibit minimal amino acid sequence homology to that of BRK and Src family kinases (Fig 4.10). This may be reflective of divergent substrate specificites and/or catalytic properties that may aid in explaining, for instance, the reduced level of total tyrosine phosphorylated protein targets of SRMS compared to BRK. We propose to employ structural studies on full length wild type SRMS, its functional domains and specific mutants cloned as GST-fused constructs and expressed in a eukaryotic system.

It is possible that besides Y380 as the site for autophosphorylation, other tyrosine residues are also phosphorylated on SRMS. Mass spectrometry analyses may be employed to deduce the tyrosine residues phosphorylated on SRMS.

6.4 Investigating the cellular roles of SRMS

The present study describes the biochemical characterization of the novel non-receptor tyrosine kinase, SRMS as well as the characterization of Dok1 as its substrate. Considering that the biochemical and cellular characteristics of this protein tyrosine kinase remained unknown since 1994, the results described in this study shall invoke interest to investigate the potential role of SRMS in regulating cellular processes and perhaps breast tumorigenesis. This is especially interesting since the other two members of the FRK/PTK6 family kinases have been shown to play opposing roles in breast cancer with BRK portraying an oncogenic function contrasting with the tumor-suppressive functions of FRK.

The stimulus for investigating the functional role of SRMS stems from the observation that unlike BRK and FRK, whose kinase activities are tightly controlled upon phosphorylation of the negative-regulatory tyrosine residue in their C-termini, SRMSlacks such a regulatory C-terminus region and appears to be constitutively active when ectopically expressed. From the biochemical analyses reported in this study, it is now known that SRMS is not negatively-regulated like BRK or FRK but that it apparently exists in a conformationally active state in cells. The physiological ramifications shall be investigated in our future studies.

We have also characterized Dok1 as a substrate of SRMS. Dok1 is functionally characterized by a pleckstrin homology domain (PH) that allows anchorage to the membrane, a phosphotyrosine binding domain that is involved in protein-protein interaction, and a C-terminal region rich in tyrosine, proline and serine residues (Cong *et al.*, 1999). Even as the candidate tumor suppressor protein has been shown to inhibit cell proliferation and transformation, it also promotes cell spreading and cell migration (Hosooka *et al.*, 2001). Dok1 is a substrate of a variety of receptor and non-receptor tyrosine kinases including the Src tyrosine kinase family members Tec and Bcr-Abl. Such tyrosine phosphorylation of Dok1 has been implicated in the regulation of its cellular functions (Gerard et al., 2004; Lee et al., 2004b; Liang et al., 2002; Noguchi et al., 1999a; Woodring et al., 2004b). Niu *et al.* characterized the nuclear export signal (NLS) embedded within the C-terminal segment of Dok1 and demonstrated that phosphorylation via Src prevents the entry of Dok1 into the nucleus, thereby regulating its sub-cellular localization and functions (Niu *et al.*, 2006). More recently, Dok1 has been shown to undergo ubiquitin mediated cellular degradation upon phosphorylation by oncogenic tyrosine kinases, v-Src and BCR-ABL (Janas and Van Aelst, 2011). It will be interesting to investigate how SRMS regulates the functions of Dok1

via phosphorylation. Along these lines, it will be interesting to identify other substrates/binding partners of SRMS via mass spectrometry analyses in conjunction with the identification of tyrosine phosphorylated residues on SRMS.

Overall, this study establishes SRMS as a potential non-receptor tyrosine kinase involved in breast tumorigenesis. This is supported by (a) the results from Fig 4.1 that SRMS, like BRK, is potentially overexpressed in the majority of breast cancer cell lines studied and (b) the identification of a proposed tumor-suppressor protein, Dok1, as its physiological substrate. This work is significant because it paves the way for further research to identify additional factors involved in the regulation of SRMS expression and activity, and to determine how SRMS regulates the cellular role of Dok1. Furthermore, unlike other similar tyrosine kinases, the atypical mechanism of catalytic regulation exhibited by SRMS hints towards a possibly divergent mode of cellular function. We, therefore, acknowledge the significance of directing subsequent investigations towards unravelling the cellular functions of this protein tyrosine kinase in breast cancer. Thus, for future studies, it may be important to determine the functional and possibly the diagnostic, prognostic and therapeutic significance of SRMS expression in breast cancer.

BIBLIOGRAPHY

Akerblom, B., Anneren, C., and Welsh, M. (2007). A role of FRK in regulation of embryonal pancreatic beta cell formation. Mol Cell Endocrinol *270*, 73-78.

Anderson, S.K., Gibbs, C.P., Tanaka, A., Kung, H.J., and Fujita, D.J. (1985). Human cellular src gene: nucleotide sequence and derived amino acid sequence of the region coding for the carboxy-terminal two-thirds of pp60c-src. Mol Cell Biol *5*, 1122-1129.

Anneren, C. (2002). Dual role of the tyrosine kinase GTK and the adaptor protein SHB in beta-cell growth: enhanced beta-cell replication after 60% pancreatectomy and increased sensitivity to streptozotocin. J Endocrinol *172*, 145-153.

Anneren, C., Lindholm, C.K., Kriz, V., and Welsh, M. (2003). The FRK/RAK-SHB signaling cascade: a versatile signal-transduction pathway that regulates cell survival, differentiation and proliferation. Curr Mol Med *3*, 313-324.

Anneren, C., Reedquist, K.A., Bos, J.L., and Welsh, M. (2000). GTK, a Src-related tyrosine kinase, induces nerve growth factor-independent neurite outgrowth in PC12 cells through activation of the Rap1 pathway. Relationship to Shb tyrosine phosphorylation and elevated levels of focal adhesion kinase. J Biol Chem *275*, 29153-29161.

Anneren, C., and Welsh, M. (2000). Role of the Bsk/Iyk non-receptor tyrosine kinase for the control of growth and hormone production in RINm5F cells. Growth Factors *17*, 233-247.

Anneren, C., and Welsh, M. (2001). Increased cytokine-induced cytotoxicity of pancreatic islet cells from transgenic mice expressing the Src-like tyrosine kinase GTK. Mol Med *7*, 301-310.

Anneren, C., and Welsh, M. (2002). GTK tyrosine kinase-induced alteration of IRS-protein signalling in insulin producing cells. Mol Med *8*, 705-713.

Anneren, C., Welsh, M., and Jansson, L. (2007). Glucose intolerance and reduced islet blood flow in transgenic mice expressing the FRK tyrosine kinase under the control of the rat insulin promoter. Am J Physiol Endocrinol Metab *292*, E1183-1190.

Armistead, P.M., and Thorp, H.H. (2002). Electrochemical detection of gene expression in tumor samples: overexpression of Rak nuclear tyrosine kinase. Bioconjug Chem *13*, 172-176.

Aubele, M., Auer, G., Walch, A.K., Munro, A., Atkinson, M.J., Braselmann, H., Fornander, T., and Bartlett, J.M. (2007). PTK (protein tyrosine kinase)-6 and HER2 and 4, but not HER1 and 3 predict long-term survival in breast carcinomas. Br J Cancer *96*, 801-807.

Aubele, M., Vidojkovic, S., Braselmann, H., Ritterswurden, D., Auer, G., Atkinson, M.J., Tapio, S., Hofler, H., Rauser, S., and Bartlett, J.M. (2009). Overexpression of PTK6 (breast tumor kinase) protein--a prognostic factor for long-term breast cancer survival--is not due to gene amplification. Virchows Arch *455*, 117-123.

Barker, K.T., Jackson, L.E., and Crompton, M.R. (1997). BRK tyrosine kinase expression in a high proportion of human breast carcinomas. Oncogene *15*, 799-805.

Becher, R., Gibas, Z., Karakousis, C., and Sandberg, A.A. (1983a). Nonrandom chromosome changes in malignant melanoma. Cancer Res *43*, 5010-5016.

Becher, R., Gibas, Z., and Sandberg, A.A. (1983b). Chromosome 6 in malignant melanoma. Cancer Genet Cytogenet *9*, 173-175.

Beildeck, M.E., Gelmann, E.P., and Byers, S.W. (2010). Cross-regulation of signaling pathways: an example of nuclear hormone receptors and the canonical Wnt pathway. Exp Cell Res *316*, 1763-1772.

Benedict, W.F., Murphree, A.L., Banerjee, A., Spina, C.A., Sparkes, M.C., and Sparkes, R.S. (1983). Patient with 13 chromosome deletion: evidence that the retinoblastoma gene is a recessive cancer gene. Science *219*, 973-975.

Berclaz, G., Altermatt, H.J., Rohrbach, V., Dreher, E., Ziemiecki, A., and Andres, A.C. (2000). Hormone-dependent nuclear localization of the tyrosine kinase iyk in the normal human breast epithelium and loss of expression during carcinogenesis. Int J Cancer *85*, 889-894.

Berger, A.H., Niki, M., Morotti, A., Taylor, B.S., Socci, N.D., Viale, A., Brennan, C., Szoke, J., Motoi, N., Rothman, P.B., *et al.* (2010). Identification of DOK genes as lung tumor suppressors. Nat Genet *42*, 216-223.

Besin, G., Yousefi, M., Saba, I., Klinck, R., Pandolfi, P.P., and Duplay, P. (2012). Dok-1 overexpression promotes development of gammadelta natural killer T cells. Eur J Immunol *42*, 2491-2504.

Bhargava, R., Gerald, W.L., Li, A.R., Pan, Q., Lal, P., Ladanyi, M., and Chen, B. (2005). EGFR gene amplification in breast cancer: correlation with epidermal growth factor receptor mRNA and protein expression and HER-2 status and absence of EGFR-activating mutations. Mod Pathol *18*, 1027-1033.

Bhat, A., Johnson, K.J., Oda, T., Corbin, A.S., and Druker, B.J. (1998). Interactions of p62(dok) with p210(bcr-abl) and Bcr-Abl-associated proteins. J Biol Chem *273*, 32360-32368.

Bibbins, K.B., Boeuf, H., and Varmus, H.E. (1993). Binding of the Src SH2 domain to phosphopeptides is determined by residues in both the SH2 domain and the phosphopeptides. Mol Cell Biol *13*, 7278-7287.

Boerner, R.J., Kassel, D.B., Barker, S.C., Ellis, B., DeLacy, P., and Knight, W.B. (1996). Correlation of the phosphorylation states of pp60c-src with tyrosine kinase activity: the intramolecular pY530-SH2 complex retains significant activity if Y419 is phosphorylated. Biochemistry *35*, 9519-9525.

Brauer, P.M., and Tyner, A.L. (2009). RAKing in AKT: a tumor suppressor function for the intracellular tyrosine kinase FRK. Cell Cycle *8*, 2728-2732.

Brauer, P.M., and Tyner, A.L. (2010). Building a better understanding of the intracellular tyrosine kinase PTK6 - BRK by BRK. Biochim Biophys Acta *1806*, 66-73.

Brauer, P.M., Zheng, Y., Evans, M.D., Dominguez-Brauer, C., Peehl, D.M., and Tyner, A.L. (2011). The alternative splice variant of protein tyrosine kinase 6 negatively regulates growth and enhances PTK6-mediated inhibition of beta-catenin. PLoS One *6*, e14789.

Breitenlechner, C.B., Kairies, N.A., Honold, K., Scheiblich, S., Koll, H., Greiter, E., Koch, S., Schafer, W., Huber, R., and Engh, R.A. (2005). Crystal structures of active SRC kinase domain complexes. J Mol Biol *353*, 222-231.

Briggs, S.D., and Smithgall, T.E. (1999). SH2-kinase linker mutations release Hck tyrosine kinase and transforming activities in Rat-2 fibroblasts. J Biol Chem *274*, 26579-26583.

Brognard, J., and Newton, A.C. (2008). PHLiPPing the switch on Akt and protein kinase C signaling. Trends Endocrinol Metab *19*, 223-230.

Burke, G., Hiscock, A., Klein, A., Niks, E.H., Main, M., Manzur, A.Y., Ng, J., de Vile, C., Muntoni, F., Beeson, D., *et al.* (2013). Salbutamol benefits children with congenital myasthenic syndrome due to DOK7 mutations. Neuromuscul Disord *23*, 170-175.

Cai, D., Dhe-Paganon, S., Melendez, P.A., Lee, J., and Shoelson, S.E. (2003). Two new substrates in insulin signaling, IRS5/DOK4 and IRS6/DOK5. J Biol Chem *278*, 25323-25330.

Campbell, S.J., and Jackson, R.M. (2003). Diversity in the SH2 domain family phosphotyrosyl peptide binding site. Protein Eng *16*, 217-227.

Cance, W.G., Craven, R.J., Bergman, M., Xu, L., Alitalo, K., and Liu, E.T. (1994). Rak, a novel nuclear tyrosine kinase expressed in epithelial cells. Cell Growth Differ *5*, 1347-1355.

Cance, W.G., Craven, R.J., Weiner, T.M., and Liu, E.T. (1993). Novel protein kinases expressed in human breast cancer. Int J Cancer *54*, 571-577.

Cargnello, M., and Roux, P.P. (2011). Activation and function of the MAPKs and their substrates, the MAPK-activated protein kinases. Microbiol Mol Biol Rev *75*, 50-83.

Carnero, A., Blanco-Aparicio, C., Renner, O., Link, W., and Leal, J.F. (2008). The PTEN/PI3K/AKT signalling pathway in cancer, therapeutic implications. Curr Cancer Drug Targets *8*, 187-198.

Carpino, N., Wisniewski, D., Strife, A., Marshak, D., Kobayashi, R., Stillman, B., and Clarkson, B. (1997). p62(dok): a constitutively tyrosine-phosphorylated, GAP-associated protein in chronic myelogenous leukemia progenitor cells. Cell *88*, 197-204.

Castro, N.E., and Lange, C.A. (2010). Breast tumor kinase and extracellular signal-regulated kinase 5 mediate Met receptor signaling to cell migration in breast cancer cells. Breast Cancer Res *12*, R60.

Cetkovic, H., Grebenjuk, V.A., Muller, W.E., and Gamulin, V. (2004). Src proteins/src genes: from sponges to mammals. Gene *342*, 251-261.

Chandrasekharan, S., Qiu, T.H., Alkharouf, N., Brantley, K., Mitchell, J.B., and Liu, E.T. (2002). Characterization of mice deficient in the Src family nonreceptor tyrosine kinase Frk/rak. Mol Cell Biol *22*, 5235-5247.

Chen, H.Y., Shen, C.H., Tsai, Y.T., Lin, F.C., Huang, Y.P., and Chen, R.H. (2004). Brk activates rac1 and promotes cell migration and invasion by phosphorylating paxillin. Mol Cell Biol *24*, 10558-10572.

Chen, J.S., Hung, W.S., Chan, H.H., Tsai, S.J., and Sun, H.S. (2013). In silico identification of oncogenic potential of fyn-related kinase in hepatocellular carcinoma. Bioinformatics *29*, 420-427.

Chen, T., Boisvert, F.M., Bazett-Jones, D.P., and Richard, S. (1999). A role for the GSG domain in localizing Sam68 to novel nuclear structures in cancer cell lines. Mol Biol Cell *10*, 3015-3033.

Cong, F., Yuan, B., and Goff, S.P. (1999). Characterization of a novel member of the DOK family that binds and modulates Abl signaling. Molecular and Cellular Biology *19*, 8314-8325.

Cooper, J.A., Gould, K.L., Cartwright, C.A., and Hunter, T. (1986). Tyr527 is phosphorylated in pp60c-src: implications for regulation. Science *231*, 1431-1434.

Corbi-Verge, C., Marinelli, F., Zafra-Ruano, A., Ruiz-Sanz, J., Luque, I., and Faraldo-Gomez, J.D. (2013). Two-state dynamics of the SH3-SH2 tandem of Abl kinase and the allosteric role of the N-cap. Proc Natl Acad Sci U S A *110*, E3372-3380.

Cowan-Jacob, S.W., Fendrich, G., Manley, P.W., Jahnke, W., Fabbro, D., Liebetanz, J., and Meyer, T. (2005). The crystal structure of a c-Src complex in an active conformation suggests possible steps in c-Src activation. Structure *13*, 861-871.

Craven, R.J., Cance, W.G., and Liu, E.T. (1995). The nuclear tyrosine kinase Rak associates with the retinoblastoma protein pRb. Cancer Res *55*, 3969-3972.

Curtis, R.E., Xiang, J., Parikh, A., Kinnaird, P., and Xing, E.P. (2012). Enabling dynamic network analysis through visualization in TVNViewer. BMC Bioinformatics *13*, 204.

Dal Col, J., and Dolcetti, R. (2008). GSK-3beta inhibition: at the crossroad between Akt and mTOR constitutive activation to enhance cyclin D1 protein stability in mantle cell lymphoma. Cell Cycle *7*, 2813-2816.

de Oliveira, G.A., Pereira, E.G., Ferretti, G.D., Valente, A.P., Cordeiro, Y., and Silva, J.L. (2013). Intramolecular dynamics within the N-Cap-SH3-SH2 regulatory unit of the c-Abl tyrosine kinase reveal targeting to the cellular membrane. J Biol Chem.

Derry, J.J., Prins, G.S., Ray, V., and Tyner, A.L. (2003). Altered localization and activity of the intracellular tyrosine kinase BRK/Sik in prostate tumor cells. Oncogene *22*, 4212-4220.

Derry, J.J., Richard, S., Carvajal, H.V., Ye, X., Vasioukhin, V., Cochrane, A.W., Chen, T., and Tyner, A.L. (2000a). Sik (BRK) phosphorylates Sam68 in the nucleus and negatively regulates its RNA binding activity. Mol. Cell. Biol. *20*, 6114-6126.

Derry, J.J., Richard, S., Valderrama Carvajal, H., Ye, X., Vasioukhin, V., Cochrane, A.W., Chen, T., and Tyner, A.L. (2000b). Sik (BRK) phosphorylates Sam68 in the nucleus and negatively regulates its RNA binding ability. Mol Cell Biol *20*, 6114-6126.

Di Cristofano, A., Niki, M., Zhao, M., Karnell, F.G., Clarkson, B., Pear, W.S., Van Aelst, L., and Pandolfi, P.P. (2001). p62(dok), a negative regulator of Ras and mitogen-activated protein kinase (MAPK) activity, opposes leukemogenesis by p210(bcr-abl). J Exp Med *194*, 275-284.

Erpel, T., Superti-Furga, G., and Courtneidge, S.A. (1995). Mutational analysis of the Src SH3 domain: the same residues of the ligand binding surface are important for intra- and intermolecular interactions. EMBO J *14*, 963-975.

Fadare, O., and Tavassoli, F.A. (2008). Clinical and pathologic aspects of basal-like breast cancers. Nat Clin Pract Oncol *5*, 149-159.

Fawal, M., Jean-Jean, O., Vanzo, N., and Morello, D. (2011). Novel mRNA-containing cytoplasmic granules in ALK-transformed cells. Mol Biol Cell *22*, 726-735.

Feng, S., Chen, J.K., Yu, H., Simon, J.A., and Schreiber, S.L. (1994). Two binding orientations for peptides to the Src SH3 domain: development of a general model for SH3-ligand interactions. Science *266*, 1241-1247.

Feng, S., Kasahara, C., Rickles, R.J., and Schreiber, S.L. (1995). Specific interactions outside the proline-rich core of two classes of Src homology 3 ligands. Proc Natl Acad Sci U S A *92*, 12408-12415.

Gao, Y., Cimica, V., and Reich, N.C. (2012). Suppressor of cytokine signaling 3 inhibits breast tumor kinase activation of STAT3. J Biol Chem *287*, 20904-20912.

Geier, F., Fengos, G., and Iber, D. (2011). A computational analysis of the dynamic roles of talin, Dok1, and PIPKI for integrin activation. PLoS One *6*, e24808.

Georgescu, M.M. (2010). PTEN Tumor Suppressor Network in PI3K-Akt Pathway Control. Genes Cancer *1*, 1170-1177.

Gerard, A., Favre, C., Garcon, F., Nemorin, J.G., Duplay, P., Pastor, S., Collette, Y., Olive, D., and Nunes, J.A. (2004). Functional interaction of RasGAP-binding proteins Dok-1 and Dok-2 with the Tec protein tyrosine kinase. Oncogene *23*, 1594-1598.

Gerard, A., Ghiotto, M., Fos, C., Guittard, G., Compagno, D., Galy, A., Lemay, S., Olive, D., and Nunes, J.A. (2009). Dok-4 is a novel negative regulator of T cell activation. J Immunol *182*, 7681-7689.

Gierut, J.J., Mathur, P.S., Bie, W., Han, J., and Tyner, A.L. (2012). Targeting protein tyrosine kinase 6 enhances apoptosis of colon cancer cells following DNA damage. Mol Cancer Ther *11*, 2311-2320.

Grimm, J., Sachs, M., Britsch, S., Di Cesare, S., Schwarz-Romond, T., Alitalo, K., and Birchmeier, W. (2001). Novel p62dok family members, dok-4 and dok-5, are substrates of the c-Ret receptor tyrosine kinase and mediate neuronal differentiation. J Cell Biol *154*, 345-354.

Grucza, R.A., Bradshaw, J.M., Futterer, K., and Waksman, G. (1999). SH2 domains: from structure to energetics, a dual approach to the study of structure-function relationships. Med Res Rev *19*, 273-293.

Guiu, S., Michiels, S., Andre, F., Cortes, J., Denkert, C., Di Leo, A., Hennessy, B.T., Sorlie, T., Sotiriou, C., Turner, N., *et al.* (2012). Molecular subclasses of breast cancer: how do we define them? The IMPAKT 2012 Working Group Statement. Ann Oncol *23*, 2997-3006.

Haegebarth, A., Bie, W., Yang, R., Crawford, S.E., Vasioukhin, V., Fuchs, E., and Tyner, A.L. (2006). Protein tyrosine kinase 6 negatively regulates growth and promotes enterocyte differentiation in the small intestine. Mol Cell Biol *26*, 4949-4957.

Haegebarth, A., Heap, D., Bie, W., Derry, J.J., Richard, S., and Tyner, A.L. (2004). The nuclear tyrosine kinase BRK/Sik phosphorylates and inhibits the RNA-binding activities of the Sam68-like mammalian proteins SLM-1 and SLM-2. J Biol Chem *279*, 54398-54404.

Haegebarth, A., Nunez, R., and Tyner, A.L. (2005). The intracellular tyrosine kinase Brk sensitizes non-transformed cells to inducers of apoptosis. Cell Cycle *4*, 1239-1246.

Haegebarth, A., Perekatt, A.O., Bie, W., Gierut, J.J., and Tyner, A.L. (2009). Induction of protein tyrosine kinase 6 in mouse intestinal crypt epithelial cells promotes DNA damage-induced apoptosis. Gastroenterology *137*, 945-954.

Hanks, S.K., and Hunter, T. (1995). Protein kinases 6. The eukaryotic protein kinase superfamily: kinase (catalytic) domain structure and classification. FASEB J *9*, 576-596.

Harvey, A.J., and Crompton, M.R. (2004). The Brk protein tyrosine kinase as a therapeutic target in cancer: opportunities and challenges. Anticancer Drugs *15*, 107-111.

Harvey, A.J., Pennington, C.J., Porter, S., Burmi, R.S., Edwards, D.R., Court, W., Eccles, S.A., and Crompton, M.R. (2009). Brk protects breast cancer cells from autophagic cell death induced by loss of anchorage. Am J Pathol *175*, 1226-1234.

Hayashi, M., and Lee, J.D. (2004). Role of the BMK1/ERK5 signaling pathway: lessons from knockout mice. J Mol Med (Berl) *82*, 800-808.

Hebenstreit, D., Horejs-Hoeck, J., and Duschl, A. (2005). JAK/STAT-dependent gene regulation by cytokines. Drug News Perspect *18*, 243-249.

Hong, E., Shin, J., Bang, E., Kim, M.H., Lee, S.T., and Lee, W. (2001). Complete sequence-specific 1H, 13C and 15N resonance assignments of the human PTK6 SH2 domain. J Biomol NMR *19*, 291-292.

Hong, E., Shin, J., Kim, H.I., Lee, S.T., and Lee, W. (2004). Solution structure and backbone dynamics of the non-receptor protein-tyrosine kinase-6 Src homology 2 domain. J Biol Chem *279*, 29700-29708.

Hooker, E., Baldwin, C., and Lemay, S. (2012). New insights into Dok-4 PTB domain structure and function. Biochem Biophys Res Commun *427*, 67-72.

Hosooka, T., Noguchi, T., Nagai, H., Horikawa, T., Matozaki, T., Ichihashi, M., and Kasuga, M. (2001). Inhibition of the motility and growth of B16F10 mouse melanoma cells by dominant negative mutants of Dok-1. Mol Cell Biol *21*, 5437-5446.

Hosoya, N., Qiao, Y., Hangaishi, A., Wang, L., Nannya, Y., Sanada, M., Kurokawa, M., Chiba, S., Hirai, H., and Ogawa, S. (2005). Identification of a SRC-like tyrosine kinase gene, FRK, fused with ETV6 in a patient with acute myelogenous leukemia carrying a t(6;12)(q21;p13) translocation. Genes Chromosomes Cancer *42*, 269-279.

Ie Kim, H., and Lee, S.T. (2009). Oncogenic functions of PTK6 are enhanced by its targeting to plasma membrane but abolished by its targeting to nucleus. J Biochem *146*, 133-139.

Ikeda, O., Miyasaka, Y., Sekine, Y., Mizushima, A., Muromoto, R., Nanbo, A., Yoshimura, A., and Matsuda, T. (2009). STAP-2 is phosphorylated at tyrosine-250 by Brk and modulates Brk-mediated STAT3 activation. Biochem Biophys Res Commun *384*, 71-75.

Ikeda, O., Mizushima, A., Sekine, Y., Yamamoto, C., Muromoto, R., Nanbo, A., Oritani, K., Yoshimura, A., and Matsuda, T. (2011). Involvement of STAP-2 in Brk-mediated phosphorylation and activation of STAT5 in breast cancer cells. Cancer Sci *102*, 756-761.

Ikeda, O., Sekine, Y., Mizushima, A., Nakasuji, M., Miyasaka, Y., Yamamoto, C., Muromoto, R., Nanbo, A., Oritani, K., Yoshimura, A., *et al.* (2010). Interactions of STAP-2 with Brk and STAT3 participate in cell growth of human breast cancer cells. J Biol Chem *285*, 38093-38103.

Irie, H.Y., Shrestha, Y., Selfors, L.M., Frye, F., Iida, N., Wang, Z., Zou, L., Yao, J., Lu, Y., Epstein, C.B., *et al.* (2010). PTK6 regulates IGF-1-induced anchorage-independent survival. PLoS One *5*, e11729.

Janas, J.A., and Van Aelst, L. (2011). Oncogenic tyrosine kinases target Dok-1 for ubiquitin-mediated proteasomal degradation to promote cell transformation. Mol Cell Biol *31*, 2552-2565.

Jin, L., and Craven, R.J. (2013). The Rak/Frk tyrosine kinase associates with and internalizes the epidermal growth factor receptor. Oncogene.

Kamalati, T., Jolin, H.E., Fry, M.J., and Crompton, M.R. (2000). Expression of the BRK tyrosine kinase in mammary epithelial cells enhances the coupling of EGF signalling to PI 3-kinase and Akt, via erbB3 phosphorylation. Oncogene *19*, 5471-5476.

Kamalati, T., Jolin, H.E., Mitchell, P.J., Barker, K.T., Jackson, L.E., Dean, C.J., Page, M.J., Gusterson, B.A., and Crompton, M.R. (1996). Brk, a breast tumor-derived non-receptor protein-tyrosine kinase, sensitizes mammary epithelial cells to epidermal growth factor. J Biol Chem *271*, 30956-30963.

Kang, K.N., Kim, M., Pae, K.M., and Lee, S.T. (2002). Characterization of the 5'-flanking region of the human PTK6 gene. Biochim Biophys Acta *1574*, 365-369.

Kang, S.A., Lee, E.S., Yoon, H.Y., Randazzo, P.A., and Lee, S.T. (2010). PTK6 inhibits down-regulation of EGF receptor through phosphorylation of ARAP1. J Biol Chem *285*, 26013-26021.

Kaplan, P.L., Simon, S., Cartwright, C.A., and Eckhart, W. (1987). cDNA cloning with a retrovirus expression vector: generation of a pp60c-src cDNA clone. J Virol *61*, 1731-1734.

Kasprzycka, M., Majewski, M., Wang, Z.J., Ptasznik, A., Wysocka, M., Zhang, Q., Marzec, M., Gimotty, P., Crompton, M.R., and Wasik, M.A. (2006). Expression and oncogenic role of Brk (PTK6/Sik) protein tyrosine kinase in lymphocytes. Am J Pathol *168*, 1631-1641.

Kawachi, Y., Nakauchi, H., and Otsuka, F. (1997). Isolation of a cDNA encoding a tyrosine kinase expressed in murine skin. Exp Dermatol *6*, 140-146.

Kawamata, A., Inoue, A., Miyajima, D., Hemmi, H., Mashima, R., Hayata, T., Ezura, Y., Amagasa, T., Yamanashi, Y., and Noda, M. (2011). Dok-1 and Dok-2 deficiency induces osteopenia via activation of osteoclasts. J Cell Physiol *226*, 3087-3093.

Kim, H., Jung, J., Lee, E.S., Kim, Y.C., Lee, W., and Lee, S.T. (2007). Molecular dissection of the interaction between the SH3 domain and the SH2-Kinase Linker region in PTK6. Biochem Biophys Res Commun *362*, 829-834.

Kim, H., and Lee, S.T. (2005). An intramolecular interaction between SH2-kinase linker and kinase domain is essential for the catalytic activity of protein-tyrosine kinase-6. J Biol Chem *280*, 28973-28980.

Kmiecik, T.E., and Shalloway, D. (1987). Activation and suppression of pp60c-src transforming ability by mutation of its primary sites of tyrosine phosphorylation. Cell *49*, 65-73.

Ko, S., Ahn, K.E., Lee, Y.M., Ahn, H.C., and Lee, W. (2009). Structural basis of the auto-inhibition mechanism of nonreceptor tyrosine kinase PTK6. Biochem Biophys Res Commun *384*, 236-242.

Kohmura, N., Yagi, T., Tomooka, Y., Oyanagi, M., Kominami, R., Takeda, N., Chiba, J., Ikawa, Y., and Aizawa, S. (1994). A novel nonreceptor tyrosine kinase, Srm: cloning and targeted disruption. Mol Cell Biol *14*, 6915-6925.

Koo, B.K., Kim, M.H., Lee, S.T., and Lee, W. (2002). Purification and spectroscopic characterization of the human protein tyrosine kinase-6 SH3 domain. J Biochem Mol Biol *35*, 343-347.

Krebs, D.L., and Hilton, D.J. (2001). SOCS proteins: negative regulators of cytokine signaling. Stem Cells *19*, 378-387.

LaFevre-Bernt, M., Sicheri, F., Pico, A., Porter, M., Kuriyan, J., and Miller, W.T. (1998). Intramolecular regulatory interactions in the Src family kinase Hck probed by mutagenesis of a conserved tryptophan residue. J Biol Chem *273*, 32129-32134.

Le Beau, M.M., Westbrook, C.A., Diaz, M.O., and Rowley, J.D. (1985). c-src is consistently conserved in the chromosomal deletion (20q) observed in myeloid disorders. Proc Natl Acad Sci U S A *82*, 6692-6696.

Lee, C.M., Jung, I.D., Noh, K.T., Lee, J.S., Park, J.W., Heo, D.R., Park, J.H., Chang, J.H., Choi, I.W., Kim, J.S., *et al.* (2012). An essential regulatory role of downstream of kinase-1 in the ovalbumin-induced murine model of asthma. PLoS One *7*, e34554.

Lee, H., Kim, M., Lee, K.H., Kang, K.N., and Lee, S.T. (1998). Exon-intron structure of the human PTK6 gene demonstrates that PTK6 constitutes a distinct family of non-receptor tyrosine kinase. Mol Cells *8*, 401-407.

Lee, J., Wang, Z., Luoh, S.M., Wood, W.I., and Scadden, D.T. (1994). Cloning of FRK, a novel human intracellular SRC-like tyrosine kinase-encoding gene. Gene *138*, 247-251.

Lee, S., Andrieu, C., Saltel, F., Destaing, O., Auclair, J., Pouchkine, V., Michelon, J., Salaun, B., Kobayashi, R., Jurdic, P., *et al.* (2004a). IkappaB kinase beta phosphorylates Dok1 serines in response to TNF, IL-1, or gamma radiation. Proc Natl Acad Sci U S A *101*, 17416-17421.

Lee, S., Roy, F., Galmarini, C.M., Accardi, R., Michelon, J., Viller, A., Cros, E., Dumontet, C., and Sylla, B.S. (2004b). Frameshift mutation in the Dok1 gene in chronic lymphocytic leukemia. Oncogene *23*, 2287-2297.

Lee, S.T., Strunk, K.M., and Spritz, R.A. (1993). A survey of protein tyrosine kinase mRNAs expressed in normal human melanocytes. Oncogene *8*, 3403-3410.

Li, W., Shi, L., You, Y., Gong, Y., Yin, B., Yuan, J., and Peng, X. (2010). Downstream of tyrosine kinase/docking protein 6, as a novel substrate of tropomyosin-related kinase C receptor, is involved in neurotrophin 3-mediated neurite outgrowth in mouse cortex neurons. BMC Biol *8*, 86.

Li, X., Lu, Y., Liang, K., Hsu, J.M., Albarracin, C., Mills, G.B., Hung, M.C., and Fan, Z. (2012). Brk/PTK6 sustains activated EGFR signaling through inhibiting EGFR degradation and transactivating EGFR. Oncogene *31*, 4372-4383.

Liang, X.Q., Wisniewski, D., Strife, A., Shivakrupa, Clarkson, B., and Resh, M.D. (2002). Phosphatidylinositol 3-kinase and Src family kinases are required for phosphorylation and

membrane recruitment of Dok-1 in c-Kit signaling. Journal of Biological Chemistry *277*, 13732-13738.

Lim, W.A., Richards, F.M., and Fox, R.O. (1994). Structural determinants of peptide-binding orientation and of sequence specificity in SH3 domains. Nature *372*, 375-379.

Liu, X., Brodeur, S.R., Gish, G., Songyang, Z., Cantley, L.C., Laudano, A.P., and Pawson, T. (1993). Regulation of c-Src tyrosine kinase activity by the Src SH2 domain. Oncogene *8*, 1119-1126.

Liu, X.K., Zhang, X.R., Zhong, Q., Li, M.Z., Liu, Z.M., Lin, Z.R., Wu, D., and Zeng, M.S. (2013). Low expression of PTK6/Brk predicts poor prognosis in patients with laryngeal squamous cell carcinoma. J Transl Med *11*, 59.

Llor, X., Serfas, M.S., Bie, W., Vasioukhin, V., Polonskaia, M., Derry, J., Abbott, C.M., and Tyner, A.L. (1999). BRK/Sik expression in the gastrointestinal tract and in colon tumors. Clin Cancer Res *5*, 1767-1777.

Lofgren, K.A., Ostrander, J.H., Housa, D., Hubbard, G.K., Locatelli, A., Bliss, R.L., Schwertfeger, K.L., and Lange, C.A. (2011). Mammary gland specific expression of Brk/PTK6 promotes delayed involution and tumor formation associated with activation of p38 MAPK. Breast Cancer Res *13*, R89.

Lukong, K.E., Huot, M.E., and Richard, S. (2009). BRK phosphorylates PSF promoting its cytoplasmic localization and cell cycle arrest. Cell Signal *21*, 1415-1422.

Lukong, K.E., Larocque, D., Tyner, A.L., and Richard, S. (2005). Tyrosine phosphorylation of sam68 by breast tumor kinase regulates intranuclear localization and cell cycle progression. J Biol Chem *280*, 38639-38647.

Lukong, K.E., and Richard, S. (2008). Breast tumor kinase BRK requires kinesin-2 subunit KAP3A in modulation of cell migration. Cell Signal *20*, 432-442.

Luttrell, L.M., Hawes, B.E., van Biesen, T., Luttrell, D.K., Lansing, T.J., and Lefkowitz, R.J. (1996). Role of c-Src tyrosine kinase in G protein-coupled receptor- and Gbetagamma subunit-mediated activation of mitogen-activated protein kinases. J Biol Chem *271*, 19443-19450.

Ma, S., Bao, J.Y., Kwan, P.S., Chan, Y.P., Tong, C.M., Fu, L., Zhang, N., Tong, A.H., Qin, Y.R., Tsao, S.W., *et al.* (2012). Identification of PTK6, via RNA sequencing analysis, as a suppressor of esophageal squamous cell carcinoma. Gastroenterology *143*, 675-686 e671-612.

Manning, B.D., and Cantley, L.C. (2007). AKT/PKB signaling: navigating downstream. Cell *129*, 1261-1274.

Mashima, R., Hishida, Y., Tezuka, T., and Yamanashi, Y. (2009). The roles of Dok family adapters in immunoreceptor signaling. Immunol Rev *232*, 273-285.

Mayer, B.J., Hirai, H., and Sakai, R. (1995). Evidence That Sh2 Domains Promote Processive Phosphorylation by Protein-Tyrosine Kinases. Current Biology *5*, 296-305.

McPherson, R.A., Taylor, M.M., Hershey, E.D., and Sturgill, T.W. (2000). A different function for a critical tryptophan in c-Raf and Hck. Oncogene *19*, 3616-3622.

Mercier, P.L., Bachvarova, M., Plante, M., Gregoire, J., Renaud, M.C., Ghani, K., Tetu, B., Bairati, I., and Bachvarov, D. (2011). Characterization of DOK1, a candidate tumor suppressor gene, in epithelial ovarian cancer. Mol Oncol *5*, 438-453.

Meyer, T., Xu, L., Chang, J., Liu, E.T., Craven, R.J., and Cance, W.G. (2003). Breast cancer cell line proliferation blocked by the Src-related Rak tyrosine kinase. Int J Cancer *104*, 139-146.

Miah, S., Martin, A., and Lukong, K.E. (2012). Constitutive activation of breast tumor kinase accelerates cell migration and tumor growth in vivo. Oncogenesis *1*, e11.

Mihrshahi, R., and Brown, M.H. (2010). Downstream of tyrosine kinase 1 and 2 play opposing roles in CD200 receptor signaling. J Immunol *185*, 7216-7222.

Mitchell, P.J., Barker, K.T., Martindale, J.E., Kamalati, T., Lowe, P.N., Page, M.J., Gusterson, B.A., and Crompton, M.R. (1994). Cloning and characterisation of cDNAs encoding a novel non-receptor tyrosine kinase, brk, expressed in human breast tumours. Oncogene *9*, 2383-2390.

Mitchell, P.J., Barker, K.T., Shipley, J., and Crompton, M.R. (1997). Characterisation and chromosome mapping of the human non receptor tyrosine kinase gene, brk. Oncogene *15*, 1497-1502.

Mitchell, P.J., Sara, E.A., and Crompton, M.R. (2000). A novel adaptor-like protein which is a substrate for the non-receptor tyrosine kinase, BRK. Oncogene *19*, 4273-4282.

Miyake, Z., Takekawa, M., Ge, Q., and Saito, H. (2007). Activation of MTK1/MEKK4 by GADD45 through induced N-C dissociation and dimerization-mediated trans autophosphorylation of the MTK1 kinase domain. Mol Cell Biol *27*, 2765-2776.

Mizushima, N., Yoshimori, T., and Levine, B. (2010). Methods in mammalian autophagy research. Cell *140*, 313-326.

Musacchio, A., Noble, M., Pauptit, R., Wierenga, R., and Saraste, M. (1992). Crystal structure of a Src-homology 3 (SH3) domain. Nature *359*, 851-855.

Nada, S., Okada, M., MacAuley, A., Cooper, J.A., and Nakagawa, H. (1991). Cloning of a complementary DNA for a protein-tyrosine kinase that specifically phosphorylates a negative regulatory site of p60c-src. Nature *351*, 69-72.

Nagar, B., Hantschel, O., Young, M.A., Scheffzek, K., Veach, D., Bornmann, W., Clarkson, B., Superti-Furga, G., and Kuriyan, J. (2003). Structural basis for the autoinhibition of c-Abl tyrosine kinase. Cell *112*, 859-871.

Neet, K., and Hunter, T. (1995). The nonreceptor protein-tyrosine kinase CSK complexes directly with the GTPase-activating protein-associated p62 protein in cells expressing v-Src or activated c-Src. Mol Cell Biol *15*, 4908-4920.

Neet, K., and Hunter, T. (1996). Vertebrate non-receptor protein-tyrosine kinase families. Genes Cells *1*, 147-169.

Neve, R.M., Chin, K., Fridlyand, J., Yeh, J., Baehner, F.L., Fevr, T., Clark, L., Bayani, N., Coppe, J.P., Tong, F., *et al.* (2006). A collection of breast cancer cell lines for the study of functionally distinct cancer subtypes. Cancer Cell *10*, 515-527.

Niki, M., Di Cristofano, A., Zhao, M., Honda, H., Hirai, H., Van Aelst, L., Cordon-Cardo, C., and Pandolfi, P.P. (2004). Role of Dok-1 and Dok-2 in leukemia suppression. J Exp Med *200*, 1689-1695.

Niu, Y., Roy, F., Saltel, F., Andrieu-Soler, C., Dong, W., Chantegrel, A.L., Accardi, R., Thepot, A., Foiselle, N., Tommasino, M., *et al.* (2006). A nuclear export signal and phosphorylation regulate Dok1 subcellular localization and functions. Mol Cell Biol *26*, 4288-4301.

Noguchi, T., Matozaki, T., Inagaki, K., Tsuda, M., Fukunaga, K., Kitamura, Y., Kitamura, T., Shii, K., Yamanashi, Y., and Kasuga, M. (1999a). Tyrosine phosphorylation of p62(Dok) induced by cell adhesion and insulin: possible role in cell migration. Embo Journal *18*, 1748-1760.

Noguchi, T., Matozaki, T., Inagaki, K., Tsuda, M., Fukunaga, K., Kitamura, Y., Kitamura, T., Shii, K., Yamanashi, Y., and Kasuga, M. (1999b). Tyrosine phosphorylation of p62(Dok) induced by cell adhesion and insulin: possible role in cell migration. EMBO J *18*, 1748-1760.

Oberg-Welsh, C., Anneren, C., and Welsh, M. (1998). Mutation of C-terminal tyrosine residues Y497/Y504 of the Src-family member Bsk/Iyk decreases NIH3T3 cell proliferation. Growth Factors *16*, 111-124.

Oberg-Welsh, C., and Welsh, M. (1995). Cloning of BSK, a murine FRK homologue with a specific pattern of tissue distribution. Gene *152*, 239-242.

Okada, K., Inoue, A., Okada, M., Murata, Y., Kakuta, S., Jigami, T., Kubo, S., Shiraishi, H., Eguchi, K., Motomura, M., *et al.* (2006). The muscle protein Dok-7 is essential for neuromuscular synaptogenesis. Science *312*, 1802-1805.

Okada, M., Howell, B.W., Broome, M.A., and Cooper, J.A. (1993). Deletion of the SH3 domain of Src interferes with regulation by the phosphorylated carboxyl-terminal tyrosine. J Biol Chem *268*, 18070-18075.

Okada, M., Nada, S., Yamanashi, Y., Yamamoto, T., and Nakagawa, H. (1991). CSK: a protein-tyrosine kinase involved in regulation of src family kinases. J Biol Chem *266*, 24249-24252.

Okada, M., and Nakagawa, H. (1989). A protein tyrosine kinase involved in regulation of pp60c-src function. J Biol Chem *264*, 20886-20893.

Oppermann, H., Levinson, A.D., Varmus, H.E., Levintow, L., and Bishop, J.M. (1979). Uninfected vertebrate cells contain a protein that is closely related to the product of the avian sarcoma virus transforming gene (src). Proc Natl Acad Sci U S A *76*, 1804-1808.

Ostrander, J.H., Daniel, A.R., Lofgren, K., Kleer, C.G., and Lange, C.A. (2007). Breast tumor kinase (protein tyrosine kinase 6) regulates heregulin-induced activation of ERK5 and p38 MAP kinases in breast cancer cells. Cancer Res *67*, 4199-4209.

Palace, J., Lashley, D., Newsom-Davis, J., Cossins, J., Maxwell, S., Kennett, R., Jayawant, S., Yamanashi, Y., and Beeson, D. (2007). Clinical features of the DOK7 neuromuscular junction synaptopathy. Brain *130*, 1507-1515.

Palka-Hamblin, H.L., Gierut, J.J., Bie, W., Brauer, P.M., Zheng, Y., Asara, J.M., and Tyner, A.L. (2010). Identification of beta-catenin as a target of the intracellular tyrosine kinase PTK6. J Cell Sci *123*, 236-245.

Park, S.H., Lee, K.H., Kim, H., and Lee, S.T. (1997). Assignment of the human PTK6 gene encoding a non-receptor protein tyrosine kinase to 20q13.3 by fluorescence in situ hybridization. Cytogenet Cell Genet *77*, 271-272.

Parker, R.C., Mardon, G., Lebo, R.V., Varmus, H.E., and Bishop, J.M. (1985). Isolation of duplicated human c-src genes located on chromosomes 1 and 20. Mol Cell Biol *5*, 831-838.

Partanen, J., Armstrong, E., Bergman, M., Makela, T.P., Hirvonen, H., Huebner, K., and Alitalo, K. (1991). cyl encodes a putative cytoplasmic tyrosine kinase lacking the conserved tyrosine autophosphorylation site (Y416src). Oncogene *6*, 2013-2018.

Pawson, T. (1994). SH2 and SH3 domains in signal transduction. Adv Cancer Res *64*, 87-110.

Pawson, T., and Gish, G.D. (1992). SH2 and SH3 domains: from structure to function. Cell *71*, 359-362.

Pawson, T., Gish, G.D., and Nash, P. (2001). SH2 domains, interaction modules and cellular wiring. Trends Cell Biol *11*, 504-511.

Pendergast, A.M. (1996). Nuclear tyrosine kinases: from Abl to WEE1. Curr Opin Cell Biol *8*, 174-181.

Pimienta, G., and Pascual, J. (2007). Canonical and alternative MAPK signaling. Cell Cycle *6*, 2628-2632.

Pluk, H., Dorey, K., and Superti-Furga, G. (2002). Autoinhibition of c-Abl. Cell *108*, 247-259.

Prat, A., Karginova, O., Parker, J.S., Fan, C., He, X., Bixby, L., Harrell, J.C., Roman, E., Adamo, B., Troester, M., *et al.* (2013). Characterization of cell lines derived from breast cancers and normal mammary tissues for the study of the intrinsic molecular subtypes. Breast Cancer Res Treat *142*, 237-255.

Qiu, H., and Miller, W.T. (2002). Regulation of the nonreceptor tyrosine kinase Brk by autophosphorylation and by autoinhibition. J Biol Chem *277*, 34634-34641.

Qiu, H., and Miller, W.T. (2004). Role of the Brk SH3 domain in substrate recognition. Oncogene *23*, 2216-2223.

Qiu, H., Zappacosta, F., Su, W., Annan, R.S., and Miller, W.T. (2005). Interaction between Brk kinase and insulin receptor substrate-4. Oncogene *24*, 5656-5664.

Radha, V., Nambirajan, S., and Swarup, G. (1996). Association of Lyn tyrosine kinase with the nuclear matrix and cell-cycle-dependent changes in matrix-associated tyrosine kinase activity. Eur J Biochem *236*, 352-359.

Rajakulendran, T., Sahmi, M., Lefrancois, M., Sicheri, F., and Therrien, M. (2009). A dimerization-dependent mechanism drives RAF catalytic activation. Nature *461*, 542-545.

Regan, C.P., Li, W., Boucher, D.M., Spatz, S., Su, M.S., and Kuida, K. (2002). Erk5 null mice display multiple extraembryonic vascular and embryonic cardiovascular defects. Proc Natl Acad Sci U S A *99*, 9248-9253.

Rimawi, M.F., Shetty, P.B., Weiss, H.L., Schiff, R., Osborne, C.K., Chamness, G.C., and Elledge, R.M. (2010). Epidermal growth factor receptor expression in breast cancer association with biologic phenotype and clinical outcomes. Cancer *116*, 1234-1242.

Robinson, D.R., Wu, Y.M., and Lin, S.F. (2000). The protein tyrosine kinase family of the human genome. Oncogene *19*, 5548-5557.

Robinson, D.R., Zylstra, C.R., and Williams, B.O. (2008). Wnt signaling and prostate cancer. Curr Drug Targets *9*, 571-580.

Roskoski, R., Jr. (2004). Src protein-tyrosine kinase structure and regulation. Biochem Biophys Res Commun *324*, 1155-1164.

Sabe, H., Knudsen, B., Okada, M., Nada, S., Nakagawa, H., and Hanafusa, H. (1992). Molecular cloning and expression of chicken C-terminal Src kinase: lack of stable association with c-Src protein. Proc Natl Acad Sci U S A *89*, 2190-2194.

Saulnier, A., Vaissiere, T., Yue, J., Siouda, M., Malfroy, M., Accardi, R., Creveaux, M., Sebastian, S., Shahzad, N., Gheit, T., *et al.* (2012). Inactivation of the putative suppressor gene DOK1 by promoter hypermethylation in primary human cancers. Int J Cancer *130*, 2484-2494.

Sawyer, T.K. (1998). Src homology-2 domains: structure, mechanisms, and drug discovery. Biopolymers *47*, 243-261.

Schlessinger, J., and Ullrich, A. (1992). Growth factor signaling by receptor tyrosine kinases. Neuron *9*, 383-391.

Seidel-Dugan, C., Meyer, B.E., Thomas, S.M., and Brugge, J.S. (1992). Effects of SH2 and SH3 deletions on the functional activities of wild-type and transforming variants of c-Src. Mol Cell Biol *12*, 1835-1845.

Serfas, M.S., and Tyner, A.L. (2003). Brk, Srm, Frk, and Src42A form a distinct family of intracellular Src-like tyrosine kinases. Oncol Res *13*, 409-419.

Shen, C.H., Chen, H.Y., Lin, M.S., Li, F.Y., Chang, C.C., Kuo, M.L., Settleman, J., and Chen, R.H. (2008). Breast tumor kinase phosphorylates p190RhoGAP to regulate rho and ras and promote breast carcinoma growth, migration, and invasion. Cancer Res *68*, 7779-7787.

Sheng, Z.M., Marchetti, A., Buttitta, F., Champeme, M.H., Campani, D., Bistocchi, M., Lidereau, R., and Callahan, R. (1996). Multiple regions of chromosome 6q affected by loss of heterozygosity in primary human breast carcinomas. Br J Cancer *73*, 144-147.

Shi, L., Yue, J., You, Y., Yin, B., Gong, Y., Xu, C., Qiang, B., Yuan, J., Liu, Y., and Peng, X. (2006). Dok5 is substrate of TrkB and TrkC receptors and involved in neurotrophin induced MAPK activation. Cell Signal *18*, 1995-2003.

Shi, N., Ye, S., Bartlam, M., Yang, M., Wu, J., Liu, Y., Sun, F., Han, X., Peng, X., Qiang, B., *et al.* (2004). Structural basis for the specific recognition of RET by the Dok1 phosphotyrosine binding domain. J Biol Chem *279*, 4962-4969.

Sicheri, F., Moarefi, I., and Kuriyan, J. (1997). Crystal structure of the Src family tyrosine kinase Hck. Nature *385*, 602-609.

Siouda, M., Yue, J., Shukla, R., Guillermier, S., Herceg, Z., Creveaux, M., Accardi, R., Tommasino, M., and Sylla, B.S. (2012). Transcriptional regulation of the human tumor suppressor DOK1 by E2F1. Mol Cell Biol *32*, 4877-4890.

Smart, J.E., Oppermann, H., Czernilofsky, A.P., Purchio, A.F., Erikson, R.L., and Bishop, J.M. (1981). Characterization of sites for tyrosine phosphorylation in the transforming protein of Rous sarcoma virus (pp60v-src) and its normal cellular homologue (pp60c-src). Proc Natl Acad Sci U S A *78*, 6013-6017.

Songyang, Z., Shoelson, S.E., Chaudhuri, M., Gish, G., Pawson, T., Haser, W.G., King, F., Roberts, T., Ratnofsky, S., Lechleider, R.J., *et al.* (1993). SH2 domains recognize specific phosphopeptide sequences. Cell *72*, 767-778.

Srour, M., Bolduc, V., Guergueltcheva, V., Lochmuller, H., Gendron, D., Shevell, M.I., Poulin, C., Mathieu, J., Bouchard, J.P., and Brais, B. (2010). DOK7 mutations presenting as a proximal myopathy in French Canadians. Neuromuscul Disord *20*, 453-457.

Sunitha, I., and Avigan, M.I. (1994). A newly identified tyrosine kinase is preferentially expressed in the gastrointestinal tract. Biochim Biophys Acta *1221*, 348-352.

Sunitha, I., and Avigan, M.I. (1996). The apical membranes of maturing gut columnar epithelial cells contain the enzymatically active form of a newly identified fyn-related tyrosine kinase. Oncogene *13*, 547-559.

Superti-Furga, G., Fumagalli, S., Koegl, M., Courtneidge, S.A., and Draetta, G. (1993). Csk inhibition of c-Src activity requires both the SH2 and SH3 domains of Src. EMBO J *12*, 2625-2634.

Tajeddine, N., and Gailly, P. (2012). TRPC1 protein channel is major regulator of epidermal growth factor receptor signaling. J Biol Chem *287*, 16146-16157.

Takeda, H., Kawamura, Y., Miura, A., Mori, M., Wakamatsu, A., Yamamoto, J., Isogai, T., Matsumoto, M., Nakayama, K.I., Natsume, T., *et al.* (2010). Comparative analysis of human SRC-family kinase substrate specificity in vitro. J Proteome Res *9*, 5982-5993.

Thomas, S.M., and Brugge, J.S. (1997). Cellular functions regulated by Src family kinases. Annu Rev Cell Dev Biol *13*, 513-609.

Thuveson, M., Albrecht, D., Zurcher, G., Andres, A.C., and Ziemiecki, A. (1995). iyk, a novel intracellular protein tyrosine kinase differentially expressed in the mouse mammary gland and intestine. Biochem Biophys Res Commun *209*, 582-589.

Tian, M., and Martin, G.S. (1996). Reduced phosphotyrosine binding by the v-Src SH2 domain is compatible with wild-type transformation. Oncogene *12*, 727-734.

Tupper, J., Crompton, M.R., and Harvey, A.J. (2011). Breast tumor kinase (Brk/PTK6) plays a role in the differentiation of primary keratinocytes. Arch Dermatol Res *303*, 293-297.

Turjanski, A.G., Vaque, J.P., and Gutkind, J.S. (2007). MAP kinases and the control of nuclear events. Oncogene *26*, 3240-3253.

Tzivion, G., Dobson, M., and Ramakrishnan, G. (2011). FoxO transcription factors; Regulation by AKT and 14-3-3 proteins. Biochim Biophys Acta *1813*, 1938-1945.

Vasioukhin, V., Serfas, M.S., Siyanova, E.Y., Polonskaia, M., Costigan, V.J., Liu, B., Thomason, A., and Tyner, A.L. (1995). A novel intracellular epithelial cell tyrosine kinase is expressed in the skin and gastrointestinal tract. Oncogene *10*, 349-357.

Vasioukhin, V., and Tyner, A.L. (1997). A role for the epithelial-cell-specific tyrosine kinase Sik during keratinocyte differentiation. Proc Natl Acad Sci U S A *94*, 14477-14482.

Vieira, A.V., Lamaze, C., and Schmid, S.L. (1996). Control of EGF receptor signaling by clathrin-mediated endocytosis. Science *274*, 2086-2089.

Wang, D.S., and Shaw, G. (1995). The association of the C-terminal region of beta I sigma II spectrin to brain membranes is mediated by a PH domain, does not require membrane proteins, and coincides with a inositol-1,4,5 triphosphate binding site. Biochem Biophys Res Commun *217*, 608-615.

Wang, D.S., Shaw, R., Winkelmann, J.C., and Shaw, G. (1994). Binding of PH domains of beta-adrenergic receptor kinase and beta-spectrin to WD40/beta-transducin repeat containing regions of the beta-subunit of trimeric G-proteins. Biochem Biophys Res Commun *203*, 29-35.

Wang, L.L., Richard, S., and Shaw, A.S. (1995). P62 association with RNA is regulated by tyrosine phosphorylation. J Biol Chem *270*, 2010-2013.

Wang, T.C., Jee, S.H., Tsai, T.F., Huang, Y.L., Tsai, W.L., and Chen, R.H. (2005). Role of breast tumour kinase in the in vitro differentiation of HaCaT cells. Br J Dermatol *153*, 282-289.

Weinberg, R.A. (1995). The retinoblastoma protein and cell cycle control. Cell *81*, 323-330.

Welch, P.J., and Wang, J.Y. (1993). A C-terminal protein-binding domain in the retinoblastoma protein regulates nuclear c-Abl tyrosine kinase in the cell cycle. Cell *75*, 779-790.

Wen, J., Xia, Q., Wang, C., Liu, W., Chen, Y., Gao, J., Gong, Y., Yin, B., Ke, Y., Qiang, B., *et al.* (2009). Dok-5 is involved in cardiomyocyte differentiation through PKB/FOXO3a pathway. J Mol Cell Cardiol *47*, 761-769.

Weng, Z., Rickles, R.J., Feng, S., Richard, S., Shaw, A.S., Schreiber, S.L., and Brugge, J.S. (1995). Structure-function analysis of SH3 domains: SH3 binding specificity altered by single amino acid substitutions. Mol Cell Biol *15*, 5627-5634.

Weston, C.R., and Davis, R.J. (2002). The JNK signal transduction pathway. Curr Opin Genet Dev *12*, 14-21.

Williams, J.C., Wierenga, R.K., and Saraste, M. (1998). Insights into Src kinase functions: structural comparisons. Trends Biochem Sci *23*, 179-184.

Woodring, P.J., Meisenhelder, J., Johnson, S.A., Zhou, G.L., Field, J., Shah, K., Bladt, F., Pawson, T., Niki, M., Pandolfi, P.P., *et al.* (2004a). c-Abl phosphorylates Dok1 to promote filopodia during cell spreading. J Cell Biol *165*, 493-503.

Woodring, P.J., Meisenhelder, J., Johnson, S.A., Zhou, G.L., Field, J., Shah, K., Bladt, F., Pawson, T., Niki, M., Pandolfi, P.P., *et al.* (2004b). c-Abl phosphorylates Dok1 to promote filopodia during cell spreading. Journal of Cell Biology *165*, 493-503.

Xiang, B., Chatti, K., Qiu, H., Lakshmi, B., Krasnitz, A., Hicks, J., Yu, M., Miller, W.T., and Muthuswamy, S.K. (2008). Brk is coamplified with ErbB2 to promote proliferation in breast cancer. Proc Natl Acad Sci U S A *105*, 12463-12468.

Xu, D., and Qu, C.K. (2008). Protein tyrosine phosphatases in the JAK/STAT pathway. Front Biosci *13*, 4925-4932.

Xu, W., Doshi, A., Lei, M., Eck, M.J., and Harrison, S.C. (1999). Crystal structures of c-Src reveal features of its autoinhibitory mechanism. Mol Cell *3*, 629-638.

Xu, W., Harrison, S.C., and Eck, M.J. (1997). Three-dimensional structure of the tyrosine kinase c-Src. Nature *385*, 595-602.

Yaffe, M.B. (2002). Phosphotyrosine-binding domains in signal transduction. Nat Rev Mol Cell Biol *3*, 177-186.

Yamakawa, N., Tsuchida, K., and Sugino, H. (2002). The rasGAP-binding protein, Dok-1, mediates activin signaling via serine/threonine kinase receptors. EMBO J *21*, 1684-1694.

Yamanashi, Y., and Baltimore, D. (1997). Identification of the Abl- and rasGAP-associated 62 kDa protein as a docking protein, Dok. Cell *88*, 205-211.

Yao, L., Kawakami, Y., and Kawakami, T. (1994). The pleckstrin homology domain of Bruton tyrosine kinase interacts with protein kinase C. Proc Natl Acad Sci U S A *91*, 9175-9179.

Yasuda, T., Shirakata, M., Iwama, A., Ishii, A., Ebihara, Y., Osawa, M., Honda, K., Shinohara, H., Sudo, K., Tsuji, K., *et al.* (2004). Role of Dok-1 and Dok-2 in myeloid homeostasis and suppression of leukemia. J Exp Med *200*, 1681-1687.

Yim, E.K., Peng, G., Dai, H., Hu, R., Li, K., Lu, Y., Mills, G.B., Meric-Bernstam, F., Hennessy, B.T., Craven, R.J., *et al.* (2009a). Rak functions as a tumor suppressor by regulating PTEN protein stability and function. Cancer Cell *15*, 304-314.

Yim, E.K., Siwko, S., and Lin, S.Y. (2009b). Exploring Rak tyrosine kinase function in breast cancer. Cell Cycle *8*, 2360-2364.

Young, M.A., Gonfloni, S., Superti-Furga, G., Roux, B., and Kuriyan, J. (2001). Dynamic coupling between the SH2 and SH3 domains of c-Src and Hck underlies their inactivation by C-terminal tyrosine phosphorylation. Cell *105*, 115-126.

Yu, H., Chen, J.K., Feng, S., Dalgarno, D.C., Brauer, A.W., and Schreiber, S.L. (1994). Structural basis for the binding of proline-rich peptides to SH3 domains. Cell *76*, 933-945.

Zarubin, T., and Han, J. (2005). Activation and signaling of the p38 MAP kinase pathway. Cell Res *15*, 11-18.

Zhang, J., Gill, A.J., Issacs, J.D., Atmore, B., Johns, A., Delbridge, L.W., Lai, R., and McMullen, T.P. (2012). The Wnt/beta-catenin pathway drives increased cyclin D1 levels in lymph node metastasis in papillary thyroid cancer. Hum Pathol *43*, 1044-1050.

Zhang, P., Ostrander, J.H., Faivre, E.J., Olsen, A., Fitzsimmons, D., and Lange, C.A. (2005). Regulated association of protein kinase B/Akt with breast tumor kinase. J Biol Chem *280*, 1982-1991.

Zhang, Y., and Dong, C. (2007). Regulatory mechanisms of mitogen-activated kinase signaling. Cell Mol Life Sci *64*, 2771-2789.

Zhao, B., Tan, P.H., Li, S.S., and Pei, D. (2013). Systematic characterization of the specificity of the SH2 domains of cytoplasmic tyrosine kinases. J Proteomics *81*, 56-69.

Zhao, M., Schmitz, A.A., Qin, Y., Di Cristofano, A., Pandolfi, P.P., and Van Aelst, L. (2001). Phosphoinositide 3-kinase-dependent membrane recruitment of p62(dok) is essential for its

negative effect on mitogen-activated protein (MAP) kinase activation. J Exp Med *194*, 265-274.

Zheng, Y., Asara, J.M., and Tyner, A.L. (2012a). Protein-tyrosine kinase 6 promotes peripheral adhesion complex formation and cell migration by phosphorylating p130 CRK-associated substrate. J Biol Chem *287*, 148-158.

Zheng, Y., Gierut, J., Wang, Z., Miao, J., Asara, J.M., and Tyner, A.L. (2012b). Protein tyrosine kinase 6 protects cells from anoikis by directly phosphorylating focal adhesion kinase and activating AKT. Oncogene.

Zheng, Y., Peng, M., Wang, Z., Asara, J.M., and Tyner, A.L. (2010). Protein tyrosine kinase 6 directly phosphorylates AKT and promotes AKT activation in response to epidermal growth factor. Mol Cell Biol *30*, 4280-4292.

Zhong, J.L., Poghosyan, Z., Pennington, C.J., Scott, X., Handsley, M.M., Warn, A., Gavrilovic, J., Honert, K., Kruger, A., Span, P.N., *et al.* (2008). Distinct functions of natural ADAM-15 cytoplasmic domain variants in human mammary carcinoma. Mol Cancer Res *6*, 383-394.

Zhou, M.M., Huang, B., Olejniczak, E.T., Meadows, R.P., Shuker, S.B., Miyazaki, M., Trub, T., Shoelson, S.E., and Fesik, S.W. (1996). Structural basis for IL-4 receptor phosphopeptide recognition by the IRS-1 PTB domain. Nat Struct Biol *3*, 388-393.

Zhou, X., Hua, L., Zhang, W., Zhu, M., Shi, Q., Li, F., Zhang, L., Song, C., and Yu, R. (2012). FRK controls migration and invasion of human glioma cells by regulating JNK/c-Jun signaling. J Neurooncol *110*, 9-19.